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Title: Long Term Changes in Hormone and Carcinogen Metabolizing Enzymes Following Neonatal Exposure to Xenobiotics in the Rat

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Donald R. Buhler

Adult hepatic metabolism has been shown to be altered in rats neonatally exposed to xenobiotics. We examined the effects of neonatal exposure to diethylstilbestrol (DES), phenobarbital (PB), 7,12-dimethylbenz(a)anthracene (DMBA) and pregnenolone-16α-carbonitrile on metabolism in rats at 24 weeks of age. In males, neonatal exposure to DES produced alterations in microsomal testosterone metabolism including increased levels of the major female metabolite (7α-hydroxytestosterone) and decreased male-predominant metabolites (2α-, 16α- and 6ß-hydroxytestosterones and androstenedione). In contrast, males neonatally exposed to PB showed increased 2α-, 16α-hydroxylation and androstenedione formation but no other effects. Changes in testosterone metabolism in both groups correlated with changes in mRNA levels of P4502A1, P4502C11 or P4503A2. Western blot analysis of protein levels found that in the

DES-treated males, P4502C6 and an immunologically similar protein were increased 60 and 90% relative to control values but P4503A2 was decreased 44%. Adult males neonatally treated with PB had 150% increase in levels of anti-P4502B-reactive protein but no other significant effects. The DES- and DMBA-treated females had increased levels of the female-specific P4502C12 of 38 and 48%, respectively. Following exposure of adult males to aflatoxin B_1 (AFB_1), DNA adducts were found to be decreased by 35% in the neonatally treated DES rats. Analysis of the adducted DNA bases failed to show any changes in the relative occurrence of individual adducts. Protein concentrations of α class glutathione S-transferases (αGST) and <u>in vitro</u> conjugation of activated [^{3}H]-AFB_1, an activity associated with αGST, were both increased more than 2-fold in the DES males relative to vehicle controls. These results indicate the neonatal DES, PB and DMBA all can act to alter hepatic metabolic capabilities in adult animals. The five month delay between exposure and analysis is consistent with long-term alterations in regulatory processes rather than acute or inductive effects. There appears to be distinct differences in enzymes altered by PB and DES suggesting that these two compounds may be altering the hepatic enzyme system by different mechanisms. Neonatal DES appears to result primarily in a feminization of hepatic enzymes while neonatal PB does not cause obvious sex-related shifts in enzyme levels.

Long Term Changes in Hormone and Carcinogen Metabolizing Enzymes Following Neonatal Exposure to Xenobiotics in the Rat

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Richard C. Zangar

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Redacted for Privacy

Head of Toxicology Program

Redacted for Privacy

Dean of Graduate School

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Typed for and by Richard C. Zangar

CONTRIBUTION OF AUTHORS

Donald R. Buhler served as major advisor for my research at Oregon State University. David L. Springer of Battelle provided for my graduate stipend and contributed significant intellectual input throughout the course of the project. Jo-Ann McCrary performed the HPLC analysis of the AFB_1-DNA adduct profile while under my guidance at Battelle during the summer of 1989. Raymond F. Novak supplied the anti-αGST antibodies used in western analysis.

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INTRODUCTION

Cytochromes P450[1] are a superfamily of enzymes that occur in all phyla (Nebert et al., 1989, 1991). Approximately 20 distinct cytochrome P450 enzymes have been isolated from the rat liver (Waxman, 1988) and others probably exist. The primary function of these enzymes is to convert endogenous and foreign hydrophobic compounds to more polar metabolites that are then eliminated from the body (Guengerich, 1990, Waxman, 1988). In this role, these enzymes serve an important function in the regulation of steroid hormones and vitamins and in the elimination of drugs and other chemicals from the body. However, in some cases these enzymes create reactive intermediates that are more toxic than the parent compound. It is now widely accepted that for many toxic compounds, including genotoxic carcinogens, bioactivation by P450 is an essential step in the development of toxicity (Guengerich, 1988, 1990).

Most enzymes catalyze a specific reaction with a

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[1]Abbreviations: P450 - cytochrome P450; DES - diethylstilbestrol; PB - phenobarbital; DMBA - 7,12-dimethylbenz(a)anthracene; GH - growth hormone.

limited number of substrates. The hepatic cytochrome P450s do not fit this stereotype. A single enzyme may oxidize such diverse compounds as androgens, benzo(a)pyrene, aflatoxin B_1, aldrin, hexane, dichloroethane, nitropropane and lauric acid, to name a few (Guengerich, 1986). Furthermore, reactions catalyzed by a single enzyme may include hydrolysis, N-demethylation, denitrification, N-oxidation, epoxidation, or hydroxylation (Guengerich, 1986). There may also be several P450s that metabolize a given substrate in an identical manner. These broad-based enzyme characteristics combined with the large number of hepatic P450s make analysis of this system very complex.

Adult male and females rats show distinct differences in specific P450s (Guengerich, 1986). Steroid levels in the neonatal animal have been shown to be important in defining the specific P450 content of the adult (Skett and Gustafsson, 1979). This process has been termed "imprinting" and can be considered a part of normal development (Skett and Gustafsson, 1979). Exposure of neonatal animals to xenobiotics has been shown to alter P450 levels in adult animals in a process also referred to as imprinting (Bagley and Hayes, 1985b,c; Dieringer et al. 1980; Lamartiniere and Pardo, 1988). (The xenobiotic disruption of imprinting would be more accurately defined as altered imprinting but is typically referred to simply as an imprinting effect.) Exposure to steroid-like compounds has been shown to alter P450 enzyme activities known to be

regulated in a sex-specific manner (Dieringer et al. 1980; Jansson et al., 1985; Lamartiniere and Pardo, 1988). These effects can usually be explained by a shift from the enzyme pattern of one sex to that of the other. Other compounds, such as phenobarbital (PB), which do not have known direct hormonal effects, have also been shown to imprint the hepatic P450 system (Faris and Campbell, 1983, Bagley and Hayes, 1985b,c). Differences in study design have made it difficult to determine if nonsteroidal imprinting agents act in a similar manner as steroidal compounds.

Early imprinting studies analyzed the hepatic P450 system using substrates that were metabolized by several P450s, making it impossible to determine precisely which enzymes were altered (Bagley and Hayes, 1985a,b,c; Lamartiniere and Pardo, 1988, Dieringer et al. 1980). One of the major objectives of the current research was to address the question of which enzymes were affected by xenobiotic imprinting. Enzyme-specific metabolic assays, immunoquantitation techniques and specific mRNA probes were used to achieve this goal. Furthermore, specific analyses for a single enzyme at the level of mRNA, protein, or enzyme activity should give some insight into the level of regulation, setting the stage for future research involving changes in regulatory phenomena. Results from these analyses were used to the extent possible to indicate if barbiturates, steroids and polyaromatic hydrocarbons were acting on the same P450 forms at the same level

biochemically, thereby allowing inference as to whether similar biochemical regulatory mechanisms have been altered. I further examined the effect of these potential imprinting agents on carcinogen metabolism and DNA adduction. Previous studies have shown alterations in the number of DNA adducts following exposure to AFB_1 but did not analyze for specific enzymes that may have been responsible for these effects (Faris and Campbell, 1981, 1983; Bagley and Hayes, 1985b). I examined levels of total adduct and of specific DNA adducts following <u>in vivo</u> AFB_1 exposure and, based on these results, proceeded to analyze specific enzyme systems that I considered likely to be responsible for observed changes.

SUMMARY OF OBJECTIVES

1. To examine several known xenobiotic imprinting agents in a controlled manner so that a systematic comparison of results can be made. This approach should indicate if different classes of compounds are acting on the same enzymes in a similar fashion.

2. To extend current knowledge on the effects of xenobiotic imprinting agents from non-specific enzyme tests commonly used by other researchers (e.g. ethoxycoumarin-O-deethylase) to analyses that are enzyme specific (e.g. stereo-specific substrate metabolism, immunoquantitation and mRNA analyses).

3. To examine the effects of imprinting on the metabolism and DNA adduction of AFB_1. The analysis of specific enzyme systems known to be important in mediating AFB_1 activation and detoxification should elucidate the biochemical mechanism behind altered DNA adduction.

BACKGROUND

Nomenclature

Due to the large number of P450 enzymes, the nomenclature of this superfamily is very complex. A systematic nomenclature has been adapted (Nebert and Gonzalez, 1987, Nebert et al. 1989, 1991) and will be used here. Under this system, all nonhuman P450 genes are designated with a Cyp followed by a number to designate the family, a capital letter to designate the subfamily, and another number to designate the specific enzyme. Orthologous genes in different species would be given the same designation. A family of cytochrome P450s typically share 40% or more common amino acid sequence and within mammals, subfamilies always share >59% sequence homology. For example, Cyp1A1 would indicate the first cytochrome P450 gene that belongs to the A subfamily of the 1 family. The protein name is not so precisely defined but for the purposes of this manuscript will be derived from the gene name in the following manner. The Cyp will be changed to "P450" so that in the case above, the protein would be defined simply as P4501A1.

Table 1 provides a short summary of the characteristics of the P450 enzymes of primary concern to this study.

Regulation of Cytochrome P450 Levels in the Adult

Xenobiotic imprinting agents are believed to alter enzyme levels by modifying normal development of regulatory systems rather than by direct effects on the enzyme. In order to understand how neonatal imprinting agents could potentially alter adult P450 activities, it is necessary to understand how these enzymes are regulated in the adult. The known biochemical mechanisms of adult P450 regulation are discussed below.

The regulatory mechanisms of different P450 enzymes are very diverse and generally not fully understood. Some P450s are found in significant amounts only following induction by a foreign compound. Others are detected in only one sex or just during certain stages of development. Still other P450s do not appear to respond to known inducers and may show age or sex specificity. Induction of these enzymes can occur at every level of biochemical control (for review, see Gonzalez, 1989). Increases in transcription rate by inducing agents are common, although the presence of the inducer is believed to be necessary (Gozukara et al., 1984; Giachelli and Omiecinski, 1986,1987). Stabilization of mRNA with or without increased transcription has also been observed (e.g. P4502C11 following GH treatment or P4501A2 by 3-methylcholanthrene, respectively). Cytochrome P4503A1 has been shown to be regulated at the mRNA level but it is believed that PB may stabilize the mRNA while PCN increases

translation (Hardwick et al. 1983). Protein stability and enzyme activity may be decreased by hormone-mediated phosphorylation of P4502B1, P4502E1 and probably others (Johansson et al. 1988, Oesch-Bartlomowicz and Oesch, 1990). Protein stability may also be altered by xenobiotics such as triacetyloleandomycin (TAO) or various suicide substrates (Watkins et al., 1986; Ortiz de Montellano and Reich, 1986). Clearly, one cannot make any assumptions as to what level biochemically these enzymes are being regulated simply on the basis of altered enzyme activity.

Growth hormone (GH) has been shown to play a central role in the regulation of many of the sex-specific P450s. Male and female rats show different patterns of plasma GH concentrations after puberty (Skett and Gustafsson, 1979; Pampori et al., 1991). Growth hormone is secreted by the pituitary which is controlled by hypothalamic secretion patterns of somatostatin and GH releasing hormone (Argente et al., 1991) The male rat has peaks of plasma GH at approximately 2 hour intervals with levels dropping off to nearly zero between peaks (Pampori et al., 1991). In contrast, females have relatively constant levels of GH with small peaks and valleys occurring about every hour. Interestingly, average GH levels are similar in males and females but the differences in secretory patterns themselves are responsible for the differential response by the target tissues. Growth hormone secretion patterns appear to be responsible for many but not all of the sex differences in

hepatic P450 levels (Jansson et al., 1985; Mode et al., 1989; Waxman et al., 1985, 1988a,b, 1990). In addition, GH has been found to suppress both constitutive and induced levels of the PB-inducible P450s, P4502B1 and P4502B2, which are not sex-specific forms (Yamazoe et al., 1987) .

Developmental Regulation

Both induced and constitutive levels of several P450s are under developmental control (For reviews, see Guengerich, 1986, Waxman et al. 1985, Waxman, 1988). For example, cytochrome P4502C11 is at extremely low levels in the young of both sexes but increases dramatically in males at puberty. Cytochromes P4502C13 and P4503A2, both found at high levels in males of all ages, are present in females until puberty. In contrast, P4502C12 is found at significant levels only in the adult female. Cytochromes P4502C6 and P4502C7 are at very low levels at birth gradually increase in both sexes until adulthood, at which time both males and females have similar levels.

Imprinting

As mentioned above, a natural dichotomy exists between the sexes in several of the rat hepatic P450 enzymes that is

the result of neonatal imprinting. Early studies indicated that there was a critical period in the newborn rat where androgen exposure resulted in masculinization of the neonatal hypothalamus (Gorski, 1968). Testosterone propionate was given to newborn female rats on days 1 to 10 of age and effects on mature females were determined according to follicular development and sterility. Results indicated that after 6 days of age, the masculinizing effects of testosterone propionate were no longer present. Arai and Gorski (1968) showed that as little as 6 hours of androgen exposure during this critical period was sufficient to sterilize females. It is now known that there are sexually dimorphic nuclei in the hypothalamus and neonatal androgen is necessary for these to develop into the larger masculine form (for reviews, see Skett and Gustafsson, 1979, and Gorski et al. 1977). In young birds (and probably rats), androgen increases both neuronal survival and migration into these nuclei (Arnold and Gorski, 1984). These data suggest that the brain can be viewed as fluid or plastic during early development and that imprinting agents act to influence the final molding of the neuronal network. It is believed that these sexually dimorphic nuclei are at least partially responsible for the differences in growth hormone secretion patterns observed between male and female adult rats (Arnold and Gorski, 1984).

Although steroid-like compounds such as DES may be directly imprinting the hypothalamus, it is not clear how

other imprinting agents function. Newborn rats respond to traditional inducers such as PB (Giachelli and Omiecinski, 1986, 1987). These induced enzymes are capable of metabolizing steroids to inactive compounds and induction has been shown to result in physiologically significant changes in endogenous hormone levels (Levin et al., 1967). In the model of imprinting suggesting by these results, the imprinting agent would act to alter endogenous steroid levels and thereby indirectly imprint the hypothalamus. It has also been suggested that the CNS-depressant action of compounds such as PB may be important in the imprinting mechanism (Balazs, 1982). Again, there is little data to support this hypothesis beyond the fact that several CNS-depressants are known imprinting agents. Sutherland and Gorski (1972) did investigate the effect of PB on the masculinization of female rats by neonatal TP. They found that peripheral administration of PB inhibited "androgenization" by intrahypothalamic administration of TP but simultaneous administration of both compounds intrahypothalamically had no effect. They interpreted these results to indicate that PB was acting in a temporally different manner or at a different site than TP.

Broader Perspective

Teratology is the study of malformations resulting from exposure of the fetus to foreign compounds. A basic tenet of this field is that there are discrete stages of development when a xenobiotic may act in a unique manner to disrupt normal development and result in a malformation (Manson, 1986). At other times during development this compound will not have the same effect. By the time the animal is born most of these cellular differentiation and maturation processes are complete and this type of timed developmental effect is no longer seen for most tissues (Manson, 1986). However, the brain is an exception to this rule and it continues to form and develop through puberty. This results in the potential for the brain to be acted upon in unique, developmentally timed ways longer than most tissues. The results of this type of effect will not be gross malformations but rather subtle changes in behavior or physiological regulation. At present, there is a fair amount of study involved in the behavioral modifications associated with neonatal exposure to neuro-acting drugs. However, there currently is almost no research on the neonatal effects of these same drugs on the animal's biochemistry. There is a clear need for extension of our knowledge in this area.

Table 1. Nomenclature and characteristics of select P450 proteins.

P450	Characteristics
1A1	Trivial names: P450c, ßNF-B. Major 3-MC and TCDD inducible form. Found in most tissues and in all examined vertebrates.
1A2	Trivial names: P450d, ISF-G Inducible by isosafrole and by 3-MC and TCDD.
2A1	Trivial names: P450a, UT-F, a1, 7α, 3. Slight induction by PB or 3-MC. Testosterone 7α-hydroxylase.
2B1	Trivial names: P450b, PB-B, PB-4. Induced by structurally diverse compounds.
2B2	Trivial name: P450e, PB-D, PB-5. Similar to IIB1 in sequence and regulation.
2C6	Trivial name: PB-C, PB-1, P450k. Constitutive form that increased by PB. Shows no sex specificity.
2C7	Trivial name: P450f Constitutive form little effected by inducers. Shows no sex-specificity.
2C11	Trivial names: P450h, UT-A, M-1, 16α, 2c. Adult, male-specific form. Testosterone 16α- and 2α- hydroxylase.
2C12	Trivial names: P450i, UT-I, 15ß, 2d. Female specific in adults. Low levels in juvenile males and females.
2C13	Trivial name: P450g Male specific form in adult but not juvenile males Strongly suppressed by common P450 inducers.
2E1	Trivial name: P450j Inducible by ethanol and acetone.
3A1	Trivial names: P450p, pcn1, PB-2a, PCN-E. Inducible by PCN or PB, otherwise extremely low.
3A2	Trivial names: pcn2, 2a. Inducible by PB but not PCN or dexamethasone. Major constitutive form in adult males from young age. Females express as juveniles but not as adults.

ALTERATIONS IN TESTOSTERONE METABOLISM. ANALYSIS OF CYTOCHROME P450 ENZYME ACTIVITIES AND mRNA.

Richard C. Zangar

Donald R. Buhler

David L. Springer

Abstract

Adult patterns of testosterone metabolism have been shown to be altered in rats neonatally exposed to certain xenobiotics. We examined the effects of neonatal exposure to diethylstilbestrol (DES), phenobarbital (PB), 7,12-dimethylbenz(a)anthracene (DMBA) and pregnenolone-16α-carbonitrile (PCN) on cytochrome P450 (P450) metabolism in rats at 24 weeks of age. Hepatic microsomal testosterone metabolism was analyzed using HPLC to identify major metabolites. In males, neonatal exposure to DES produced alterations in all major testosterone metabolites including decreased (31 to 44%) 2α-, 16α-, 6ß-hydroxylations and androstenedione formation but increased (58%) 7α-hydroxylation. In contrast, males neonatally exposed to PB showed increased (20 to 27%) 2α-, 16α-hydroxylation and androstenedione formation but no effect was observed in the rate of 6ß- or 7α-hydroxylations. In order to examine potential regulatory changes at the molecular level, treatments that showed altered testosterone metabolism were further examined for changes in specific P450 mRNAs using northern blot techniques. Changes in testosterone metabolism in both groups correlated with changes in mRNA levels of P4502A1, P4502C11 or P4503A2. These results indicated that: 1) adult testosterone metabolism was modified by neonatal exposure to PB and DES in male rats and these changes were related to similar changes in mRNA levels

of specific P450s; 2) DES shifted the male pattern of testosterone-metabolizing P450s towards one that more closely resembled the female pattern; and 3) PB exposure increased P4502C11 in males independently of the other enzymes analyzed and with no indication that this change represented a sex-related shift.

Introduction

To date, at least 38 rat cytochrome P450 (P450) clones have been isolated and sequenced and these enzymes form a large superfamily of genes (Nebert et al., 1991). Many of these enzymes are expressed in the liver and are important in the oxidation of nonpolar endogenous and exogenous compounds (Guengerich, 1986, 1990). Some of these enzymes, especially P4502A1[2], P4502C11 and P4503A2, have been reported to have high testosterone metabolizing activities (Waxman, 1988). Regulation of the different P450 enzymes is complex and is only beginning to be understood. Individual P450s have been shown to be regulated at all levels, including transcription rate, RNA stability, protein stability and protein activity rates (Gonzalez, 1989; Oesch-Bartlomowicz and Oesch, 1990).

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[2]Abbreviations are P450 - cytochrome P450, DES - diethylstilbestrol, PCN - pregnenolone-16α-carbonitrile, DMBA - 7,12-dimethylbenz(a)anthracene, PB - phenobarbital.

During the neonatal period, androgens are believed to increase both neuronal survival and migration into hypothalamic nuclei resulting in sexually dimorphic nuclei in adults (Arnold and Gorski, 1984). These regions are believed to be involved in the regulation of the sexually distinct growth hormone secretion patterns exhibited in rats (Skett and Gustafsson, 1979). In turn, these secretory patterns regulate some but not all of the sex-specific P450 enzymes (Gonzalez, 1989; Jansson et al., 1985; Mode et al., 1989; Waxman et al., 1988a,b). The dichotomy in P450 expression is not apparent until puberty, when levels of some enzymes are markedly increased while others are suppressed in a manner dependant on the animal's sex (Guengerich, 1986). This developmental process of neuroendocrine regulation of hepatic enzymes has been termed "imprinting" (Skett and Gustafsson, 1979).

Not surprisingly, neonatal exposure to steroid-like compounds such as diethylstilbestrol or testosterone-propionate has been shown to alter the adult P450 pattern (Lamartiniere and Pardo, 1988; Dierenger et al., 1980). However, neonatal exposure to central nervous system depressants such as barbiturates also perturbs adult P450 levels (Haake and Safe, 1988; Bagley and Hayes, 1985b,c). Typically, these compounds also act as potent hepatic P450 inducers and it may be possible that they imprint by a mechanism distinct from that of the steroid hormones.

Neonatal treatment with benzo(a)pyrene, a polyaromatic

hydrocarbon has been shown to alter adult P450 enzyme activity (Herd and Greene, 1980). DMBA, another polyaromatic hydrocarbon, has not been investigated as an imprinting agent but was of interest because of studies that indicate it may affect the neuro-endocrine system. DMBA had estrogenic actions in vitro at nanomolar concentrations (Pasqualini et al., 1988) and it altered the in vivo cyclic excretion of 17ß-estradiol in rats in a manner that has been interpreted as a persistent alteration in the hypothalamo-pituitary function (Abed et al., 1987). PCN, a synthetic glucocorticoid and a potent P450 inducer (Hardwick et al., 1983; Graves et al., 1987), has not been tested as an imprinting agent but is similar in structure to 16α-isothiocyanopregnenalone-3-acetate that was found to be an effective imprinting agent. The combined hormonal action of PCN plus its inductive capabilities suggested that it may act as an imprinting agent.

We examined the effects of neonatal exposure to PB, DES, PCN and DMBA on the adult metabolism of testosterone. This was the first time that these agents were evaluated in a single study which allowed for the direct comparisons of results. Since the study of the xenobiotic imprinting effects had not been extended beyond enzyme activity measurements (many of which were nonspecific), we examined mRNA levels that corresponded to affected enzyme activities. Although neuroendocrine effects of these agents would be expected to be observable soon after puberty, previous

reports have indicated that differences in treated and control animals may be accentuated in older animals (Haake and Safe, 1988; Arai and Gorski, 1968). For this reason, we waited until animals were 24 weeks of age before examination.

Materials and Methods

Chemicals

The [^{14}C]testosterone and [^{32}P]ATP (specific activities of 50 mCi/mmol and 3000 Ci/mmol, respectively) were obtained from New England Nuclear. The 7α-, 6ß-, 16ß-, and 16α-hydroxytestosterone standards were from Steraloids, and 2α-hydroxytestosterone was provided courtesy of Dr. D.N. Kirk of the Steroid Reference Collection, Queen Mary College, London. Pregnenolone-16α-carbonitrile was obtained from Upjohn. Sigma provided 7,12-dimethylbenz(a)anthracene, diethylstilbestrol, sodium phenobarbital, testosterone and androstenedione.

Animals and accommodations

Female Sprague-Dawley rats ("timed pregnant") were obtained from Charles River (Raleigh, North Carolina) and individually housed under a 12 h light-dark cycle. Animals

were given free access to food and water during the course of the study. Pups were born within a 24 h period. The day of birth was defined as day 0 of age. Pups were subcutaneously dosed on days 1, 3 and 5 of age with one of the 4 test compounds or vehicle. To minimize leakage following injection, a small diameter needle was inserted above the tail of the neonatal rats and worked forward to the shoulder blades. Three males and 3 females from a single litter were exposed to one agent. An additional 3 animals of the same sex were then exposed to a different agent so that each litter contained 9 pups. Litters with less than 9 pups were supplemented with unused animals from other litters. Generally, 8 litters were used for each treatment. Adult rats were selected such that all individuals within a treatment were from different litters. Remaining animals were used in other studies.

Pups were weaned at 21 days of age and placed in group-housing with rats of the same sex and treatment. Adults were given free access to Purina Rat Chow and water. Body weights were recorded on day 1 and 1, 3, 12 and 24 weeks of age. Animals were sacrificed at 24 weeks of age.

Dose selection

A dose of 350 μg/pup/day (pups weighed about 7 g on day 1 and 15 g on day 7) was used for DES and PCN. This dose of DES was previously found to be effective in the alteration

of hepatic P450 activity (Dieringer et al 1980). A dose of 15 mg DMBA/kg/day, which may be expected to produce very few tumors in these animals (Tatken and Lewis, 1983), was used. Thirty mg PB/kg/day has been previously found to be an effective imprinting dose (Bagley and Hayes, 1985b) and was used here.

Sesame oil (Hains, cold-pressed), found to have less than 10 meq peroxide/kg oil, was used as a carrier for the DES, PCN and DMBA. Buffered saline (PBS; pH 7.4) was used as the carrier for the PB. Sesame oil or saline were administered at 3 or 6 μL/g/day, respectively.

Sample preparation and analyses

Animals were anesthetized by ip injection of sodium pentobarbital and livers were removed immediately after anesthesia. The anterior portion of the right liver lobe was removed and immediately frozen in liquid nitrogen and then stored at -80°C for later use in mRNA analyses. The remainder of the liver was used in the preparation of microsomes (Kedzierski and Buhler, 1986).

Microsomal protein concentrations were determined using the methods of Bradford (1976) with dye reagent obtained from Bio-Rad. Total cytochrome P450 was assayed using the procedures of Matsubara et al (1976). Microsomal incubation with testosterone and analysis of metabolites was performed using the procedures of van der Hoeven (1981). Twenty-four

of the 94 samples incubated were spiked with [^{14}C]-testosterone of known specific activity. Scintillation counts of fractions were used to determine the relationship between UV peak areas at 254 nm and molar concentrations of metabolite. All HPLC analyses were performed in duplicate.

mRNA analyses

Total RNA was extracted using the methods of Chomczynski and Sacchi (1987). Oligomer probes were synthesized using an Applied Biosystems 381A DNA Synthesizer. Oligonucleotide DNA sequences (24- to 30-mer) previously tested and found to be specific for P4502A1 (Matsunaga et al., 1988), P4502C11 (Yoshioka et al, 1987) and P4503A2 (Gonzalez et al., 1986a) were used. Probes were end-labelled with ^{32}P (Sambrook et al., 1989). Total RNA was loaded at 20 μg/lane onto a formaldehyde agarose (0.8%) gel and ran for 6 h at 1.5 V/cm. All samples from a single treatment and the appropriate vehicle control were individually loaded onto a single gel. The size-fractionated RNA was transferred onto a nylon membrane (GeneScreenPlus) and covalently bound prior to drying by UV activation using a Stratagene UV crosslinker set at 150,000 μJ. Membranes were hybridized overnight in 10 ng/mL of labelled probe, 1 M NaCl, 50% formamide, 1% SDS, 10% dextran sulfate, and 100 ng/mL denatured salmon-sperm DNA and washed the following morning in 0.3 M NaCl, 30 mM sodium citrate

and 1% laurylsulfate (2 X SSC, 1% SDS). Membranes were autoradiographed for 5 to 10 days using enhancer screens. Autoradiographies were analyzed using a Zeineh SL densitometer set on the "log" data integration setting. We found that this yielded approximately linear density values from 2 to 30 μg of total RNA per lane.

All significantly different results were repeatable in replicate blots. However, since density values could not be directly compared between blots, values from a single blot only are presented.

Statistics

Data were analyzed using the Mann-Whitney-Wilcoxon rank test (Mann and Whitney, 1947). Data from treated animals were compared to the appropriate vehicle control to determine the probability that change occurred in the observed direction at a significance level of $p < 0.05$.

Results

Neonatal exposure to DES resulted in reduced body weight in the males from weeks 1 to 12 of age (Fig. 1). In females, neonatal DES treatment resulted in smaller juveniles but larger animals following puberty (about 7 weeks). Growth was not affected by neonatal treatment with

PB, DMBA or PCN in either males or females. Total P450 levels were not altered in any of the treatments (data not shown).

Neonatal treatment with DMBA or PCN did not alter hepatic microsomal testosterone metabolism in adults of either sex (Fig. 2). In males, neonatal PB increased the 16α- and 2α-hydroxylations and androstenedione formation by 23, 27 and 20%, respectively, over that seen in the vehicle control group. Testosterone 7α-hydroxylation was increased 58% in the neonatally treated DES males but 16α-hydroxylation, 2α-hydroxylation and 6ß-hydroxylation and androstenedione formation were decreased to 58, 56, 60 and 69% of the vehicle control values, respectively. Neither neonatal DES nor PB altered adult female testosterone metabolism.

Observation of the data indicated that the main effect of the neonatal DES exposure on the males was to shift the testosterone metabolite profile toward that of the females. In order to statistically analyze for this trend, a multivariate significance test of the effect of DES jointly on the metabolites was carried out as follows. Using the logarithmic values of all of the data, a linear discriminant function was computed that provided the linear combination of three representative metabolites levels. Metabolites were selected that showed the greatest differences between the sexes. This function is

$$y = 0.12 \log(7\alpha) - 0.10 \log(6ß) - 0.53 \log(16\alpha)$$

where 7α, 6ß and 16α represent the appropriate testosterone hydroxylation position.

Data from individual males were then reduced to a single number y according to this formula. The effect of DES on this combined response value was significantly different from the vehicle control, as determined by the Mann-Whitney-Wilcoxon analysis, indicating that neonatal exposure to DES coordinately shifted the male hepatic microsomal testosterone metabolism to a pattern that more closely resembled the female pattern. A similar analysis of the PB testosterone data did not indicate a joint effect on the three metabolites.

Total RNA extracted from the right liver lobe of individuals from treatments which showed altered testosterone metabolism was probed using specific oligonucleotide probes. The P4502A1 probe detected 2 bands at approximately 2.0 and 3.0 kb (Fig. 3). However, more stringent washes virtually eliminated the 2.0 kb band leaving the 3.0 kb band nearly intact. The 3.0 kb band was correlated with the 7α-hydroxylase activity ($r = 0.75$, or 0.89 if one unusual value was removed from the analysis) but not the 2.0 kb band ($r = -0.18$). Nagata et al. (1987) also detected bands at 2.0 and 3.0 kb using a P4502A1 cDNA probe but concluded that the 3.0 kb band was the major P450 gene product. It is also possible that there was a weak hybridization with the 1.9 kb ribosomal RNA (Fig. 3), although no significant hybridization was seen between the

P4502A1 probe and the 4.7 kb rRNA or with the other oligomers and either of the rRNA bands. In the DES males, P4502A1 3.0 kb mRNA was increased more than two-fold over that observed in the oil controls (Fig. 4). No alteration in the 2.0 kb band was observed in either treatment. Neither band was altered in the neonatally treated PB males.

The PB-treated rats showed more than a 2 fold increase in P4502C11 mRNA levels but no change in P4503A2 levels. The P4502C11 and P4503A2 mRNAs were not significantly altered in the DES-treated male rats relative to the appropriate vehicle control (Fig. 4). Nevertheless, in the DES males the P4502C11 mRNA levels were highly correlated with the testosterone 2α- and 16α-hydroxylase activities ($r = 0.97$ and 0.98, respectively) and the P4503A2 mRNA levels correlated with the 6ß-hydroxylase activities ($r = 0.90$).

Discussion

The results indicated that neonatal DES- and PB-treatment altered testosterone metabolism in adult male rats. The 5 month delay between exposure and the measured effects was consistent with chronic changes in the regulation of these enzymes rather than acute inductive effects.

The 7α-hydroxylation of testosterone increased 58% in the DES males. The 7α-hydroxylation of testosterone has

been shown to be nearly exclusively catalyzed by P4502A1 (Levin et al., 1987). Consistent with these results, analysis of the P4502A1 mRNA indicated a 2-fold increase over the oil controls in the neonatally-exposed DES males (Fig. 4). The correlation of individual P4502A1 mRNA levels with enzyme activity further indicated that the biochemical regulation of this activity occurred at the mRNA level.

Testosterone 2α- and 16α-hydroxylations and oxidation to androstenedione were increased in PB-treated males but decreased in DES-treated males. The decrease in 16α-hydroxylase observed in the neonatally treated DES males was similar to that observed previously (Dierenger et al., 1980). The 2α- and 16α-hydroxylation products are primarily formed by the action of P4502C11 in untreated rats (Guengerich, 1986; Waxman, 1988). The 16α-hydroxylase activity may also be formed by P4502B1, an enzyme that has been shown to be very low in naive (not exposed) rats but potentially could have been present in imprinted animals. However, P4502B1 has similar levels of 16ß- and 16α-hydroxylase activities (Guengerich, 1986) but no 16ß-hydroxytestosterone was detected in the HPLC analysis of the testosterone metabolites. (Analysis of standards indicated that 16ß-hydroxytestosterone would have been clearly separated from other metabolites). This result indicates that P4502B1 was not present at levels that would contribute significantly to testosterone metabolism. Testosterone conversion to androstenedione can be catalyzed by P4502C11

but may also be formed by 17ß-hydroxysteroid oxidoreductase (Murray and Horsfield, 1990; Waxman, 1988), possibly explaining why the formation of this metabolite was not altered to the same degree as the other metabolites of P4502C11.

Northern analysis of P4502C11 mRNA showed that this mRNA was increased in neonatally treated PB males, indicating that this enzyme activity was regulated at the mRNA level. The P4502C11 mRNA in DES-treated males was not significantly decreased but we feel that regulation also occurred at the mRNA level for the following reasons. The P4502C11 mRNA levels were highly correlated with the 2α- and 16α-hydroxylase activities in the DES animals ($r = 0.97$ and 0.98, respectively) and there was a decrease in mean mRNA levels similar to that seen in the enzyme activity but was not statistically significant due to greater variability in the mRNA data.

Testosterone 6ß-hydroxylase activity was decreased in DES-treated males but not PB-treated males. P4503A2 is believed to be responsible for this activity in control rats (Guengerich, 1986; Waxman, 1988). P4503A1 may also hydroxylate at the 6ß position but this isozyme is normally not present in rats unless previously exposed to high levels of exogenous corticosteroid. Northern analysis of RNA from male DES-treated and oil control rats indicated that readily detectable levels of P4503A1 mRNA levels were present in the dexamethasone-treated sample but none was detectable in the

other samples (data not shown). Levels of P4503A2 mRNA were not statistically decreased in the DES males but, as with the P4502C11, there was a nonsignificant decrease in mean mRNA levels similar to the decrease observed for the 6ß-hydroxylase and the individual mRNA levels were strongly correlated ($r = 0.90$) with the 6ß-hydroxylase activity. We interpret these data to indicate that P4503A2 was also regulated at the mRNA level.

Metabolism of testosterone in the neonatally exposed DES males was shifted towards the female pattern. Male-specific forms (P4502C11 and P4503A2) were decreased while the major female testosterone metabolizing enzyme (P4502A1) was increased. The shift of all three of these enzymes appeared to be coordinately regulated in individual males. Thus, males which showed the greatest decrease in 16α- and 6ß-hydroxylations also showed the largest increase in 7α-hydroxylation. These results taken with the apparent regulation of all three enzymes at the mRNA level may indicate that there is a single mechanism regulating transcription in all three genes. However, the results of this study do not preclude the possibility that there was a change in the stability of one or more of the mRNAs analyzed.

Serum testosterone concentrations were assayed in the PB-treated, DES-treated and control males and were found to be significantly decreased by 42% in the DES animals but unchanged in the PB males (data not shown). No direct

correlation between testosterone levels and enzyme activity in individual DES-treated males was found indicating that factors other than testosterone were important in enzyme regulation. These results are consistent with those of Dierenger et al. (Dieringer et al., 1980) that showed that both neonatal testosterone propionate or DES treatment resulted in altered adult androstenedione 16α-hydroxylase, but only DES-exposed animals had modified testosterone levels. Possibly growth hormone secretion patterns, which are sexually dimorphic in rats and have been found to be closely correlated to the sex-specific enzyme patterns observed in rats (Pampori et al., 1991), could have been directly responsible for the changes in P450 enzymes observed in the DES-treated males.

The PB-treated males showed a response in only one testosterone metabolizing enzyme, P4502C11. As with the DES-treated males, changes in this enzyme activity were found to be associated with changes in mRNA levels. However, the lack of other sex-related shifts in testosterone-metabolizing enzymes suggests that neonatal PB treatment may have been acting in a different manner than neonatal DES. Consistent with this conclusion, Sutherland and Gorski (1972) found that intrahypothalamic injections of testosterone propionate resulted in androgenization of female rat brain. This effect could be inhibited by systemic pentobarbital but not by microinjections into the hypothalamus. They concluded that pentobarbital was either

acting at a different site than testosterone propionate or in a temporally different manner.

In contrast to reported effects following neonatal PB or DES treatment using a similar dosing protocol (Dieringer et al, 1980; Haake and Safe, 1988; Bagley and Hayes, 1985b,c), we observed no changes in P450 in neonatally treated females. The DES-treated females did show an unusual growth pattern where the animals were smaller than controls prior to sexual maturation (about 7 weeks) but then underwent a growth spurt to become significantly larger than the control females (Fig. 1). As far as we know, this type of altered growth pattern is unique. The decrease in body weight following exposure may have been due to acute toxicity of DES but the relative increase in body weight following puberty suggests that there may have been neuroendocrine changes not reflected in the hepatic enzyme analyses measured.

We did not examine specific P450 protein levels in this study. However, we feel that the combined approach of measuring enzyme activities and mRNA levels would have detected any perturbations in protein levels or activities.

Acknowledgements

The authors would like to thank Cristobal Miranda, Gary Steigler and Marilyn Henderson for their excellent technical advice and to Grant Feist and Carl Schreck for the radioimmunoassay of serum testosterone levels. This research was supported by the U.S. Department of Energy through the Northwest College and University Association of Science (Washington State University, grant DE-FG06-89ER-75522) and by NIH (grant ES-00210).

Figure 1. Growth curves for neonatally exposed male and female rats. Data points represent mean of litter means (8 or 9 litters with 3 rats/treatment/litter) except at 24 weeks, which represents the mean of the 7 or 8 animals selected for the study. For the sake of clarity, cross bars representing SE are included for the DES treatment only. Inset shows the female DES-treated body weights as a percent of the oil control. * - Body weight of DES treatment significantly different ($p < 0.05$) from oil control.

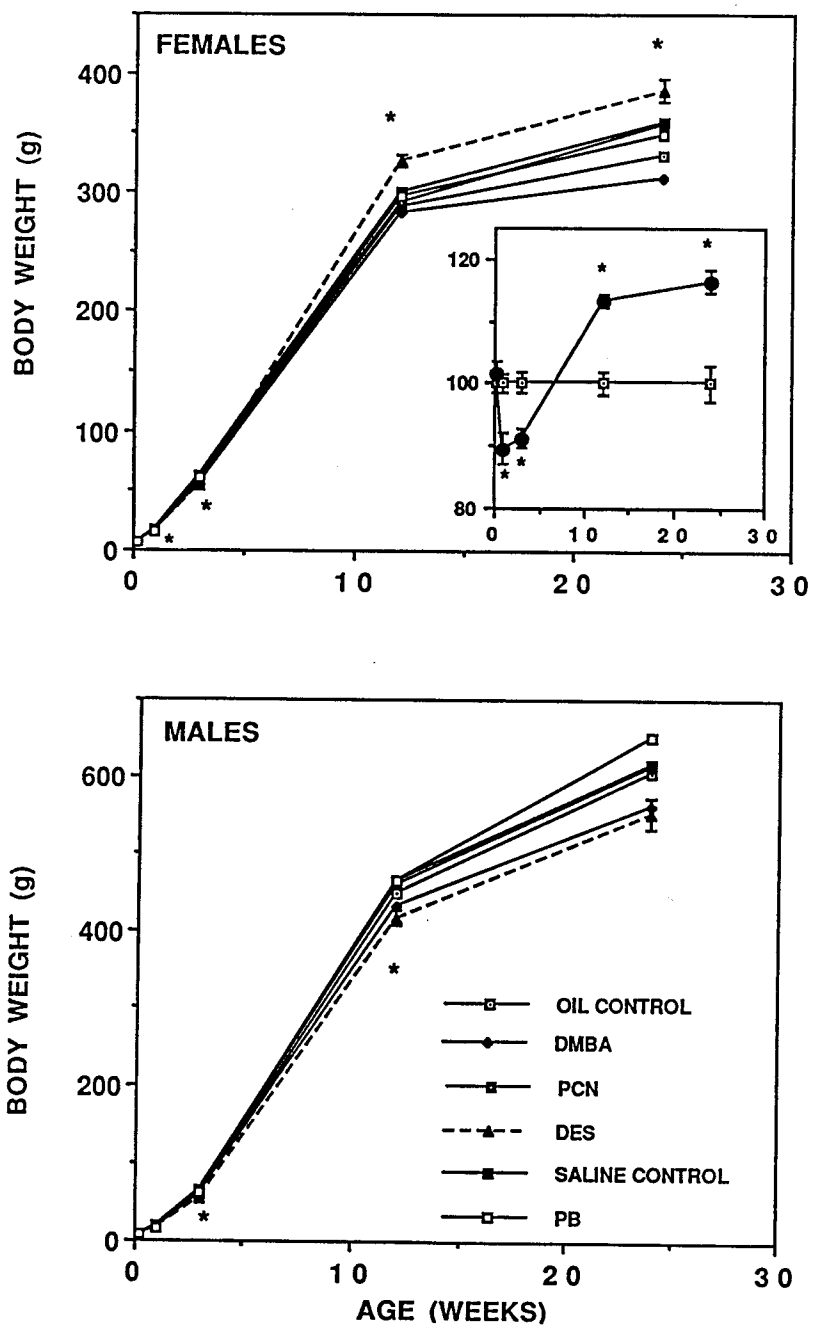
FEMALES
BODY WEIGHT (g)
400
300
200
100
0
0
10
20
30
120
100
80
MALES
600
400
200
OIL CONTROL
DMBA
PCN
DES
SALINE CONTROL
PB
AGE (WEEKS)

Figure 1

Figure 2. Hepatic microsomal metabolism of testosterone. Microsomal fractions from neonatally exposed adult rats were examined for <u>in vitro</u> ability to metabolize testosterone. Testosterone metabolites are indicated by the position of hydroxylation (e.g. 7α = 7α-hydroxytestosterone) or, for androstenedione, by an "A". The summation of the major metabolites shown is represented by "SUM". Columns and cross bars represent mean values and SE, respectively, as a percent of the appropriate vehicle control. There were 7 or 8 animals per treatment. Mean values, in pmoles/mg protein/min, for the two vehicle control groups were: males - 7α = 106, 6ß = 2281, 16α = 1398, 2α = 688, A = 677, sum = 5150; and females - 7α = 276, 6ß = 109, 16α = 54, A = 135, sum = 571.

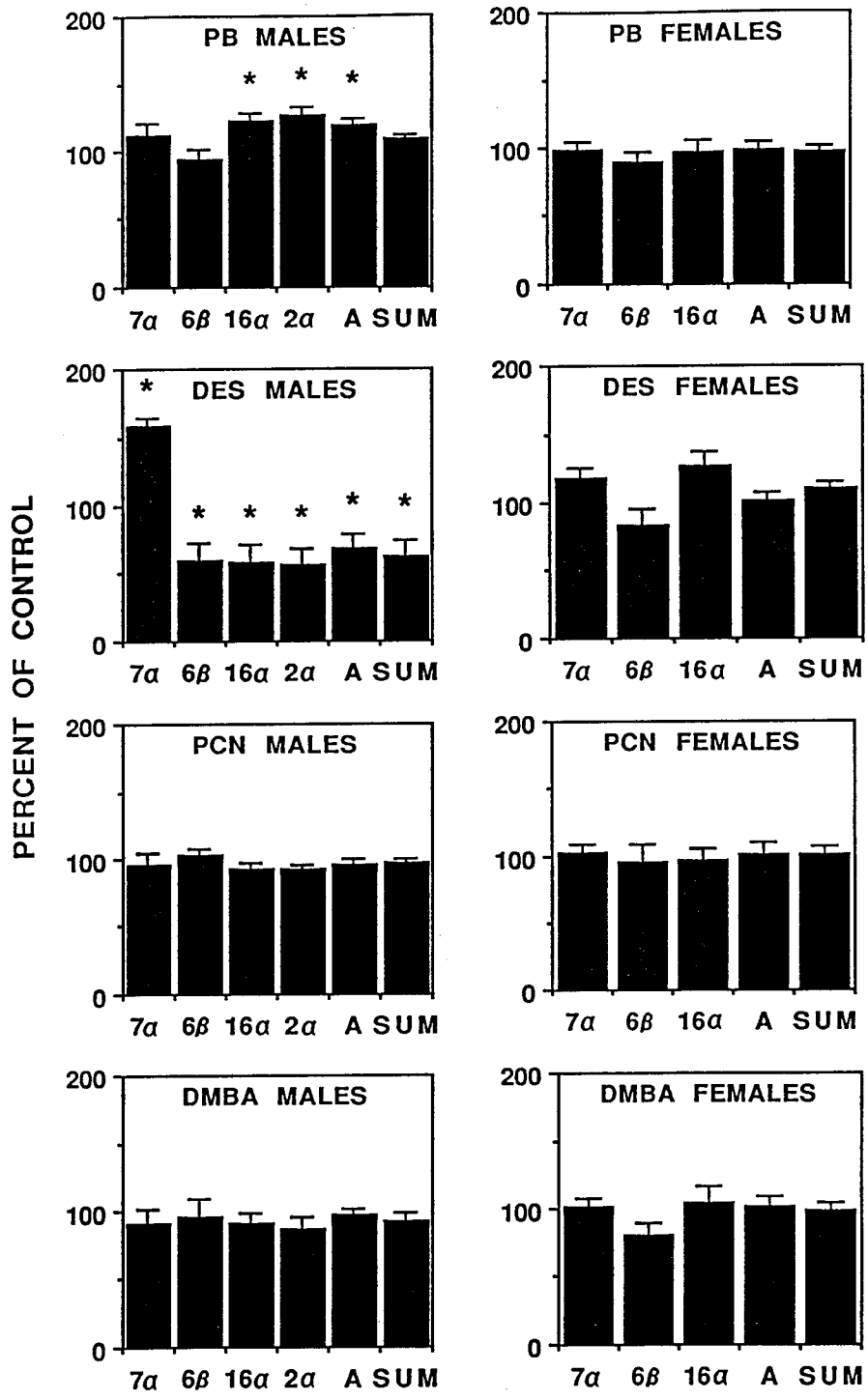
PERCENT OF CONTROL
PB MALES
PB FEMALES
DES MALES
DES FEMALES
PCN MALES
PCN FEMALES
DMBA MALES
DMBA FEMALES
200
100
0
7α 6β 16α 2α A SUM
7α 6β 16α A SUM
*

Figure 2

Figure 3. Northern blot analysis of total hepatic RNA. Samples taken from adult rats neonatally exposed to DES or vehicle. Samples were arranged so that vertically aligned lanes from different blots represent RNA from the same individual. The sample on the far left was from an untreated female. Note that individuals with the lowest levels of P4502C11 and P4503A2 have the highest levels of P4502A1. Arrowheads indicate positions of the 1.9 kb (lower) and 4.7 kb (upper) rRNAs bands. Final wash temperatures for the P4502C11, P4503A2, and low and high stringency P4502A1 blots were 48°, 55°, 42° and 52°C, respectively. See "materials and methods" for details.

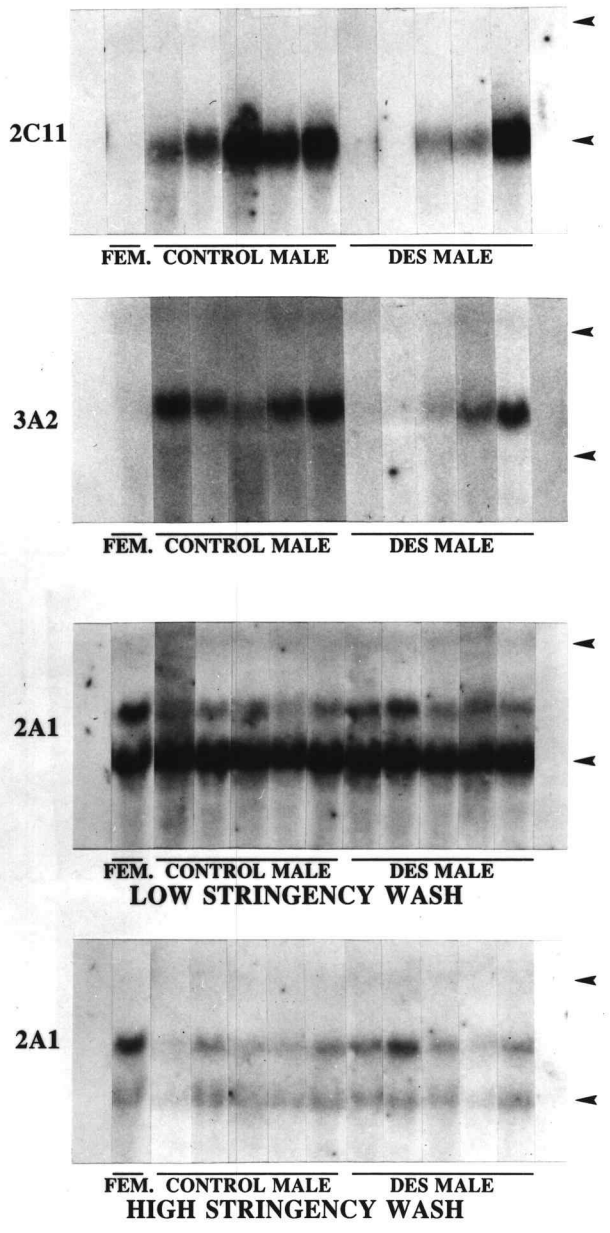
2C11
FEM.
CONTROL MALE
DES MALE
3A2
FEM.
CONTROL MALE
DES MALE
2A1
FEM.
CONTROL MALE
DES MALE
LOW STRINGENCY WASH
2A1
FEM.
CONTROL MALE
DES MALE
HIGH STRINGENCY WASH

Figure 3

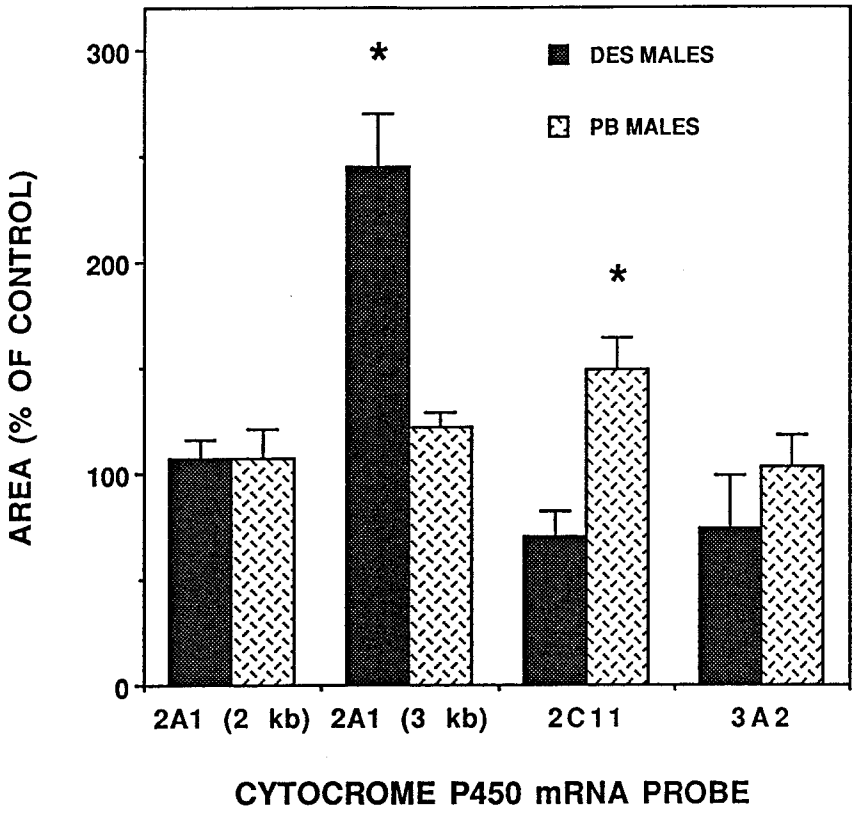

Figure 4. Densitometric analysis of northern blots in adults neonatally exposed to DES or PB. The P4502A1 oligomer probe detected 2 bands but only the 3 Kb band correlated with the testosterone 7α-hydroxylase. See text for details. Columns and cross bars represent treatment means and SE, respectively, as a percentage of the vehicle control. * - significantly different from vehicle control ($p < 0.05$).

WESTERN BLOT ANALYSIS OF CYTOCHROMES P450

Richard C. Zangar

David L. Springer

Donald R. Buhler

Abstract

Neonatal exposure to certain xenobiotics has been shown to alter hepatic metabolism in adult rats in a manner that indicates long term changes in enzyme regulation. We have previously observed changes in adult testosterone metabolism and in cytochrome P450 (P450) mRNA levels in animals neonatally exposed to PB or DES. In order to test for other enzyme alterations, we used western procedures for specific P450s to analyze archived hepatic microsomes from adult rats (24 weeks old) neonatally exposed to diethylstilbestrol (DES), phenobarbital (PB), 7,12-dimethylbenz(a)anthracene (DMBA) or pregnenolone-16α-carbonitrile (PCN). The most striking effects were observed in the DES-treated males: P4502C6 and an immunologically similar protein were increased 60 and 90% relative to control values but P4503A2 was decreased 44%. No changes were observed in the DES males in P4502E1, P4502B, or the male-specific P4502C13. Adult males neonatally treated with PB had 150% increase in levels of anti-P4502B-reactive protein but no other significant effects. The DES- and DMBA-treated females had increased levels of the female-specific P4502C12 of 38 and 48%, respectively, but no other observed alterations. The results confirm that neonatal exposure to DES or PB cause alterations in hepatic cytochrome P450 levels of adult rats but show that these chemicals act on different enzymes. Neonatal DMBA resulted in changes similar to the synthetic

estrogen, DES, in adult females but did so at about two-thirds lower dose.

Introduction

The rat cytochrome P450 (P450[3]) system is composed of at least 38 known enzymes and forms a superfamily of proteins involved in the metabolism of lipophilic compounds (Nebert et al. 1991). Many of these enzymes are expressed in rat liver and are important in the degradative metabolism of a wide variety of endogenous and exogenous compounds (Guengerich, 1986). In most cases, lipophilic compounds are oxidized to more water-soluble metabolites which are excreted directly or can be enzymatically conjugated to polar molecules that are readily eliminated. In some cases, metabolism by P450s can lead to highly reactive compounds that are extremely toxic (Guengerich, 1990).

In the rat, many of the P450s have been found to show a high degree of sex-specificity: that is, they are present at significant levels in one sex but are nearly absent in the other (Guengerich, 1986; Gonzalez, 1989). Generally, distinct differences between the sexes in individual P450

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[3]Abbreviations are P450 - cytochrome P450, DES - diethylstilbestrol, PCN - pregnenolone-16α-carbonitrile, DMBA - 7,12-dimethylbenz(a)anthracene, PB - phenobarbital, SDS-PAGE - 0.1% sodium dodecyl sulfate polyacrylamide gel electrophoresis, PBS-T - physiologically buffered saline containing 0.05% Tween-20, IgG - immunoglobin G.

levels is not observed until puberty and is believed to be associated with the onset of sex-specific patterns of growth hormone secretion (Mode et al., 1989; Guengerich, 1986; Jansson et al., 1985; Skett and Gustafsson, 1979). Long-term changes in the regulation of P450 levels resulting from chemical action and occurring only during critical stages of development has been termed imprinting (Skett and Gustafsson, 1979). Exposure in utero or within a few days of birth to estrogenic or androgenic compounds has been shown to result in alterations in hepatic metabolism in adult animals (Lamartiniere and Pardo, 1988, Dieringer et al. 1980, Jansson et al. 1985). Furthermore, these metabolic alterations are distinct from changes observed when adults are exposed to the same compound. Typically, no long term changes are observed when dosing occurs later than a few days postpartum. The delayed effect of these compounds until puberty has led to the belief that chemically induced changes in the neuroendocrine system are responsible for the alterations in the regulation of the hepatic P450 system (Skett and Gustafsson, 1979). However, neonatal exposure to compounds without known steroidal action, such as phenobarbital (PB), have also been found to be effective in altering enzyme activity (Bagley and Hayes, 1985b,c; Haake and Safe, 1988). It has been suggested that the central-nervous-system-depressant action of PB is in someway responsible for these imprinting-type effects (Balazs, 1982; Anderson et al., 1991), although this has yet

to be show experimentally. It is also possible that PB is acting through enzyme induction in the liver, which could alter endogenous steroid levels (Levin et al., 1967), or by other mechanisms.

Previous imprinting studies have investigated the effects of neonatal xenobiotics primarily by examining metabolic activity of hepatic cellular fractions. Many of these assays utilized general substrates that were not suitable for specifically determining which enzymes were being altered (Bagley and Hayes, 1985b,c; Lamartiniere and Pardo, 1988; Dieringer et al. 1980).

We had previously examined hepatic microsomal testosterone metabolism and mRNA levels in adults neonatally exposed to DES, PB, DMBA or PCN (Zangar et al, submitted). Only PB and DES were found to be active in altering P450 activities in this previous study. Using microsomes from this earlier study, we employed western blot procedures to examine levels of immunoreactive P450 protein in adult male and female rats neonatally exposed to xenobiotics. This approach allows analysis of some of the specific P450s that could be responsible for altered hepatic metabolism seen in previous studies. We focused our examination on the DES and PB treatments, but in some cases extended analyses to include the DMBA and PCN groups.

Materials and Methods

Chemicals

The PCN was obtained from Upjohn and DMBA, PCN and DES were from Sigma. Western blotting reagents were obtained from BioRad.

Animals and accommodations

Female Sprague-Dawley rats ("timed pregnant") were obtained from Charles River (Raleigh, North Carolina) and individually housed under a 12 h light-dark cycle. All litters were delivered within a 24 h period. The day of birth was defined as day 0 of age. Pups were subcutaneously dosed on days 1, 3 and 5 of age with one of the 4 test compounds or vehicle. Doses were as follows: DES and PCN - 350 μg/pup/day (pups weighed about 7 g on day 1 and 15 g on day 7); DMBA - 15 mg/kg/day; and PB - 30 mg/kg/day. Sesame oil (Hains, cold-pressed) was used as a carrier for the DES, PCN and DMBA. Physiologically buffered saline (PBS) was used as the carrier for the PB. Sesame oil or saline were administered at 3 or 6 μL/g/day, respectively.

Pups were weaned at 21 days of age and placed in group-housing with rats of the same sex and treatment. Adults were given free access to Purina Rat Chow and water. Animals were sacrificed at 24 weeks of age.

Adult animals were induced with PB, dexamethasone or ß-naphthoflavone at 50, 80, and 80 mg/kg/dy for 3 days and sacrificed on the fourth day. Hepatic microsomes prepared from these animals were used to confirm that the various antibodies would bind to induced proteins as expected.

Microsomal preparation

Animals were anesthetized by ip injection of sodium pentobarbital, livers were removed immediately after anesthesia and microsomes were prepared (Kedzierski and Buhler, 1986). Microsomal protein concentrations were determined using the methods of Bradford (1976) using dye reagent obtained from Bio-Rad. Samples were stored at -80°C until use in the current study, two to three years after initial preparation.

Antibodies

Cytochromes P4502C6 and P4502B2 were purified from male, PB-induced, hepatic microsomes using the procedures of Guengerich et al. (1982). The possibility of contaminant protein was further reduced by running purified fractions on a 16 cm SDS-PAGE gel, staining with Coomassie Blue and excising the major band. The protein from this band was then used as the antigen in the preparation of antisera in rabbits as described by Vaitukaitis (1981). The IgG

fraction was purified from the antisera by salt fractionation and DEAE-cellulose chromatography (Thomas et al., 1976)

Anti-rat-P4503A and anti-rat-P4502E1 were obtained from Oxygene (Dallas, Texas). Anti-P4502C13 was a generous gift from Dr. Joyce Goldstein.

Western blots

Microsomal protein was loaded onto a 16 or 20 cm SDS-PAGE gel (BioRad apparatus) using a 3% stacking gel and an 8% separating gel as described by Laemmli (1970). All samples from a single treatment and the appropriate controls were loaded onto a single gel. Following SDS-PAGE size fractionation, proteins bands were transferred to nitrocellulose using a BioRad electroblotter operated at 30 V for 6 hr in an aqueous solution of 25 mM Tris-base, 192 mM glycine and 20% methanol. Following transfer, nitrocellulose was dried at 70°C for 30 min, and blocked for 60 min using 5% milk powder (Carnation) in PBS containing 0.05% Tween-20 (PBS-T). Antibody solution was added to 2 to 4 μg protein/mL and allowed to incubated overnight. Blots were washed with PBS-T twice each for 30 sec and 5 min; incubated with 1:10,000 to 1:30,000 dilutions of goat-anti-rabbit horse-radish-peroxidase conjugate (BioRad) in 5% milk powder, PBS-T for 1 hr; and then washed with PBS-T twice each for 30 sec and 15 min. Incubations and washes were

performed at room temperature with gentle shaking. The enhanced chemiluminescence reagents and procedure of Amersham were used to develop Kodak X-AR5 film. The density of individual bands was determined using a Zeineh SL densitometer using the "log" data-integration setting. All statistically significant results were reproducible in replicate blots.

Statistics

Data were analyzed using Student's two-tailed t-test (Zar, 1974) at a significance level of $p < 0.05$.

Results

Densitometric analysis of the western blots resulted in approximately linear results with protein dilution over a wide range of protein concentrations and band intensities (Fig. 5). Linearity was lost if the film was overexposed but multiple exposures of each blot were taken and only those with acceptable ranges of densities were utilized.

Anti-P4503A2 did not bind to female microsomes indicating that this antibody was reacting only with the male-specific P4503A2 in these animals (Fig. 6). The P4503A2 levels were reduced in the DES males but not in other treatments (Fig. 7). This response was variable, with

some animals showing near complete loss of immunoreactive protein while others did not appear to be affected.

The anti-P4502C13 had been extensively immunopurified by Goldstein's group using procedures that had previously eliminated all detectable cross-reactivity (McClellan-Green et al., 1987). The anti-P4502C13 did not bind purified P4502C11 or P4502C6 in tests in our lab. However, there was cross-reactivity with female microsomes, which should not contain P4502C13. To determine if the anti-P4502C13 was reacting with other proteins in the males, we utilized an unique feature of the P4502C13; notably that levels of this P450 are dramatically reduced by PB and to a lesser degree by polyaromatic compounds (Bandiera et al., 1986). Test results are shown at the top of Fig 8. As seen in the first 3 lanes, untreated male microsomes show high levels of immunoreactive protein but PB-treated males contain only a trace and ßNF-treated animals show about a 50% reduction. We consider this large reduction in immunoreactive protein in males following PB exposure strong evidence that no other P450 besides P4502C13 is being detected in males with this antibody. In females, dramatically different results are obtained with the same antibody and treatments (Fig. 8). The untreated female shows high levels of immunoreactive protein but there is no decrease following PB or ßNF treatment. These results suggest that a distinct but immunologically similar protein is being detected in the females. The only major female-specific P450 reported to

date is P4502C12, which also has been shown to be immunologically similar to P4502C13 (McClellan-Green et al., 1987). Hence, we were able to use a single antibody preparation to detect 2 distinct but sex-specific proteins. The DES and DMBA females showed increases of 38 and 48% in immunodetectable protein that we believe to be P4502C12 (Fig. 7). No differences were seen in the males in P4502C13 (Fig. 7).

The anti-P4502C6 detected two distinct bands (Fig. 6). The lower band co-migrated with the purified P4502C6, was induced by PB but was not altered by ßNF or by dexamethasone, and was present at significant levels in both sexes. All of these factors are consistent with the known regulation and status of P4502C6 (Guengerich, 1986). We can not be sure of the identity of the upper band but it was not increased by PB, dexamethasone or ßNF treatment and did not show any sex specificity. Cytochrome P4502C7 is present at high levels in both sexes, is immunologically similar to P4502C6, is only weakly altered by PB, and has been shown to be regulated in parallel during with P4502C6 during development (Strazielle et al., 1991; Gonzalez et al., 1986a,b; Bandiera, 1990; Ryan et al., 1984) . The lower (P4502C6) and upper bands were increased 60 and 90%, respectively, in the DES males but were not altered in the other groups examined (Fig. 9).

The anti-P4502B detected only a single band that co-migrated with the purified standard and was highly inducible

by PB (Fig. 10). This band presumably contains both P4502B1 and P4502B2 proteins, both of which are present in untreated animals at low levels and are difficult to separate with the procedures used (Guengerich, 1986). A 2.5-fold increase in detectable protein was seen in the PB males but no change was observed in the other treatments examined (Fig. 9).

The anti-2E1 detected a single band which was not altered in any of the treatments examined (Figs. 9 and 10).

Discussion

The results indicated that neonatal DES-, PB- and DMBA-treatments altered cytochrome P450 protein levels in adult rats. The 5 month delay between exposure and the measured effects was consistent with chronic changes in the regulation of these enzymes rather than acute inductive effects.

The sex-specific enzyme P4503A2 was decreased in the neonatally treated DES males, in agreement with reduced testosterone 6ß-hydroxylase we had observed previously in these animals (Zangar et al., submitted). No change was seen in another male-specific form, P4502C13. Cytochrome P4502C13 is regulated by growth hormone secretion patterns but in a manner distinct from male-specific P450s, P4503A2 and P4502C11 (McClellan-Green et al., 1989; Zaphiropoulus et al., 1990). Male growth hormone secretion patterns induce

P4503A2 and P4502C11 but are not necessary for P4502C13 gene expression. Rather, female growth hormone patterns inhibit P4502C13 expression. It could be that some of the DES males in this study did not have typical male or female growth hormone secretion patterns, as has been observed following neonatal exposure to monosodium glutamate (Waxman et al., 1990). Cytochrome P4502C12, which was also recognized by the anti-P4502C13, has not been found in male rats unless neonatally castrated and even then estrogen or other hormonal treatment is usually required (Guengerich, 1986; Dannan et al., 1986, Kamataki et al., 1983; MacGeoch et al., 1984).

The neonatally treated DES males had increased levels of the 2 SDS-PAGE bands detected by the anti-P4502C6. The lack of sex specificity of these proteins would suggest that neither was regulated by growth hormone secretion patterns. There also was not a clear correlation between levels of P4503A2 and P4502C6 in the DES-treated males. Combined, these results suggest that there could be more than one regulatory mechanism impacted by the neonatal DES.

Neonatal exposure of females to DES also influenced adult levels of P4502C12 without altering the other P450s examined. Since this was the first change we had observed in any of the female treatments, we thought the P4502C12 might be especially susceptible to the imprinting action of xenobiotics and, hence, we extended analyses to the other treatment groups, which had not shown any changes in

previous tests (Zangar et al, submitted). The neonatal PCN treatment had no effect but DMBA-treatment resulted in increases in P4502C12 similar to that seen in the DES females (Fig. 7). Pasqualini et al. (1988) have found that DMBA may act as an estrogen at nanomolar doses in vitro. It seems likely that DMBA is acting here in a similar manner to DES, a synthetic estrogen. The DES was administered at about 3 times the dose of the DMBA, suggesting that DMBA is a potent imprinting agent in female rats.

Previously, we have observed approximately 30% and 50% increases in P4502C11 enzyme activity and mRNA levels, respectively, in neonatally treated PB males (Zangar et al., submitted). Results from the western analyses also show that neonatal PB treatment resulting in increased concentrations of P4502B protein in adult males but not in the other P450s examined. Although PB will induce P4502B1 and P4502B2 (detected as a single band by the anti-P4502B), the 5 month delay between dosing and an increase in protein levels is not consistent with a normal induction response. Gozukara et al. (1984) have shown that levels of P4502B1 and P4502B2 mRNA peaked 24 to 36 hr after a single dose of PB and returned to control levels by 5 days. We believe that the increase in P4502B was the result of long term changes in protein regulation rather than the result of normal inductive processes. The changes observed with neonatal PB were not similar to those observed with DES or DMBA. Overall, these results are not consistent with the mechanism

of imprinting by PB being the same as with DES or DMBA.

Acknowledgements

The authors would like to thank Cristobal Miranda and Marilyn Henderson for their excellent technical advice. This research was supported by NIH grant ES-00210 and by the Northwest College and University Association of Science (Washington State University) under U.S. Department of Energy grant DE-FG06-89ER-75522.

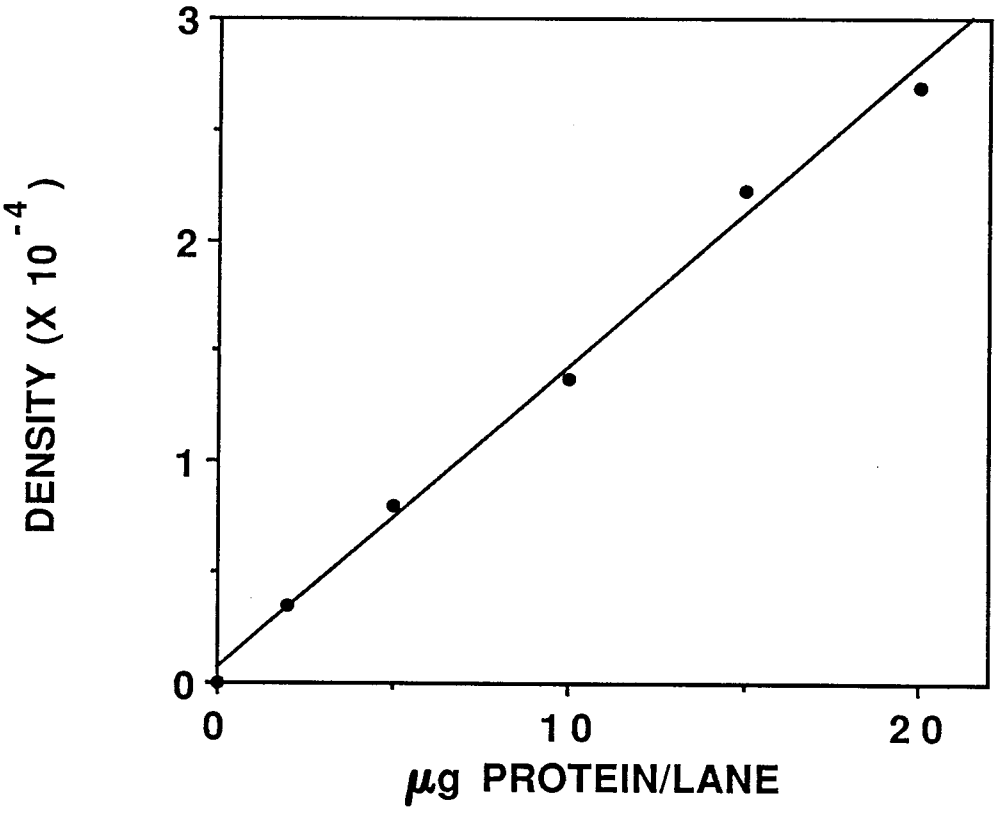

Figure 5. Standard curve of densitometry and protein levels. Analysis of a western blot prepared using male hepatic microsomes and anti-P4502E1. Two to 20 µg of microsomal protein were loaded per lane and then prepared using standard western procedures (see "materials and methods"). Values represent the average of replicate samples.

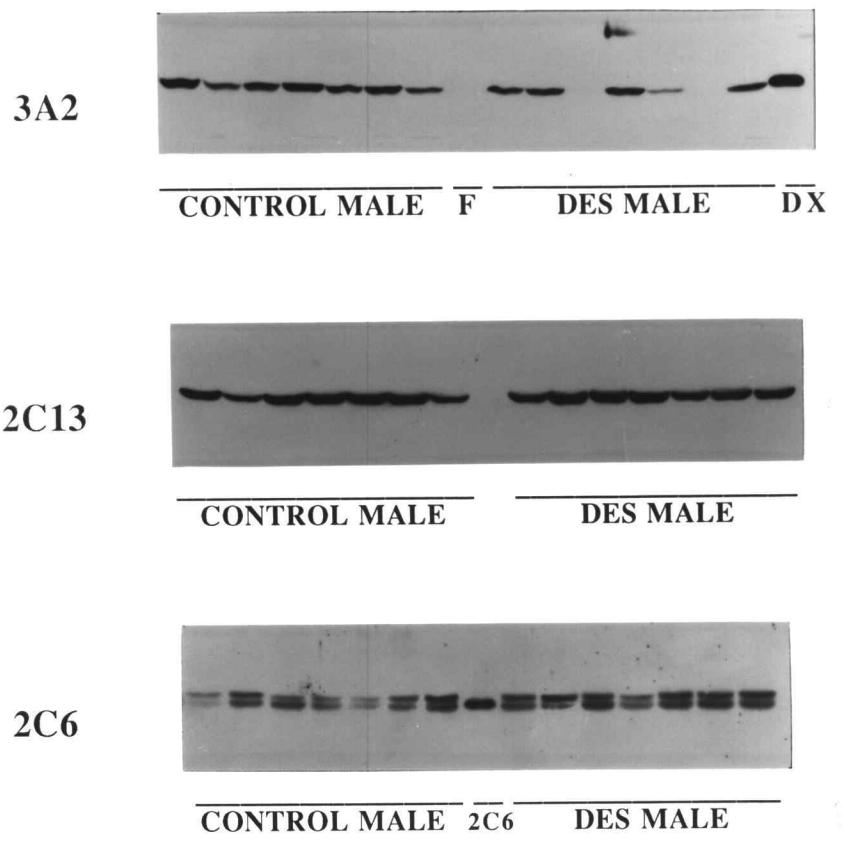

Figure 6. Western blots prepared using antibodies for cytochromes P4503A2, P4502C13 and P4502C6. Hepatic microsomes were loaded 20 μg/lane except for the dexamethasone-induced sample, which was loaded at 1 μg/lane. Key: CONTROL MALE - adult males neonatally treated with vehicle; DES MALE - adult males neonatally treated with diethylstilbestrol; F - untreated adult female, DX - adult male induced with dexamethasone for 3 consecutive days prior to sacrifice; 2C6 - purified P4502C6.

Figure 7. Densitometric analysis of western blots prepared with antibodies to P4502C13 and P4503A2. Hepatic microsomes from adult male (M) or female (F) rats that had been neonatally exposed to diethylstilbestrol (DES) dimethylbenzanthracene (DMBA), pregnenolone-16α-carbonitrile (PCN) or phenobarbital (PB) were used. Columns and cross bars represent mean and SE, respectively, of band densities as a percent of the appropriate vehicle control. *,**, *** - Significantly different from control values at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. N.D. - not determined.

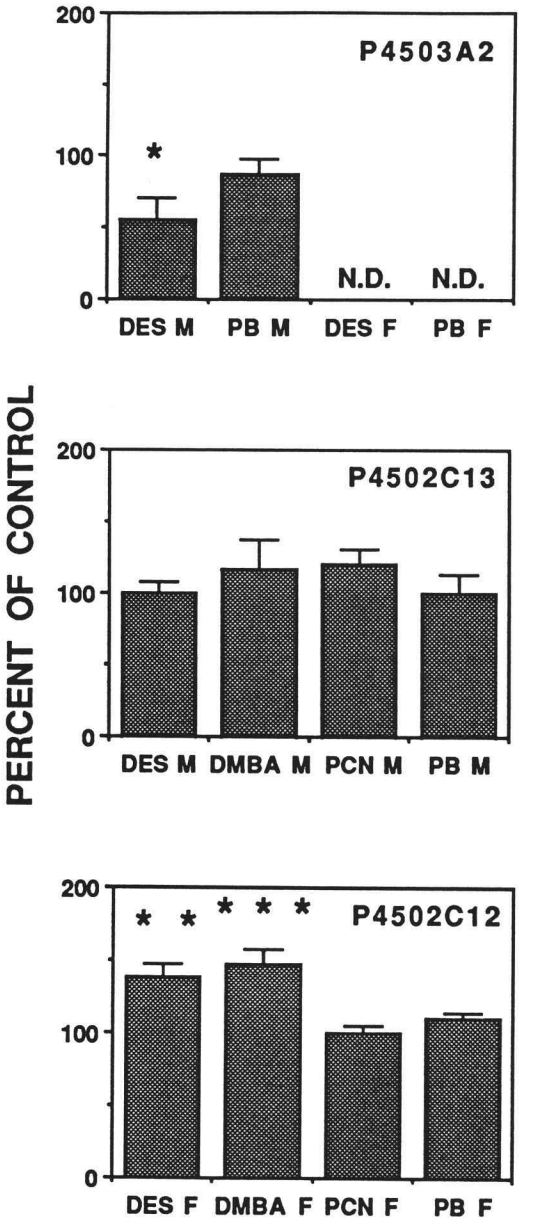
PERCENT OF CONTROL
200
100
0
P4503A2
*
N.D.
N.D.
DES M
PB M
DES F
PB F
P4502C13
DES M
DMBA M
PCN M
PB M
P4502C12
* * * * *
DES F
DMBA F
PCN F
PB F

Figure 7

Figure 8. Western blot analysis of hepatic microsomal fractions analyzed using antibodies for cytochrome P4502C13. Upper figure shows differential response following induction in adult male and female samples indicating that different P450s are being detected in each sex. Middle and lower figures show increases in immunoreactive protein detected by the anti-P4502C13 in adult females that were neonatally treated with DES or DMBA, respectively. Key: UT - untreated adult; PB - adult induced with phenobarbital for 3 consecutive days prior to sacrifice; BNF - adult animal induced with ß-naphthoflavone for 3 days prior to sacrifice; CONTROL FEMALE - adult females that were neonatally exposed to vehicle; DES FEMALE - adult females that were neonatally exposed to diethylstilbestrol; DMBA FEMALE - adult females that were neonatally exposed to dimethylbenzanthracene.

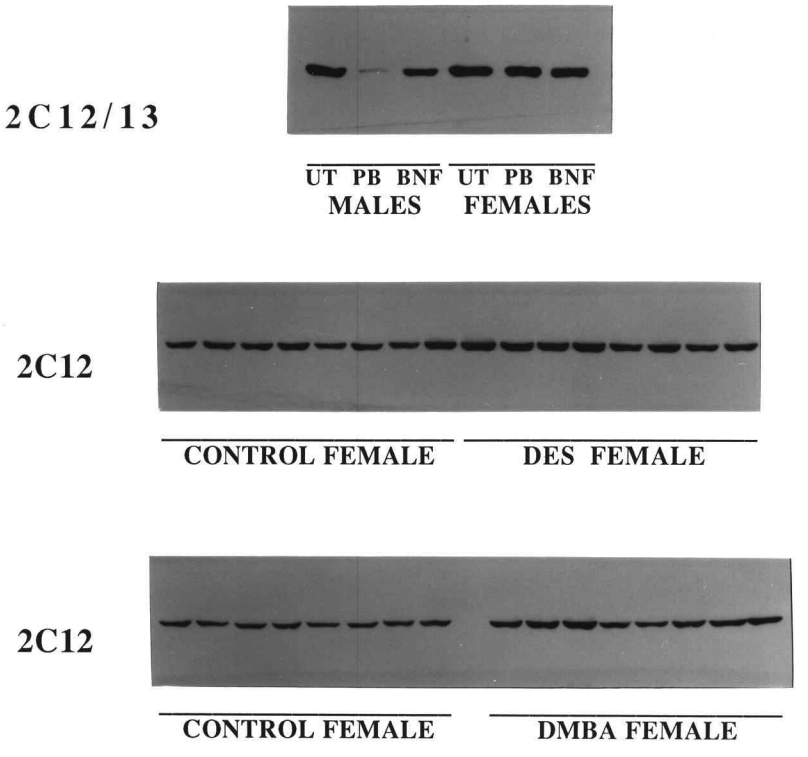
2C12/13
UT PB BNF UT PB BNF
MALES FEMALES
2C12
CONTROL FEMALE
DES FEMALE
2C12
CONTROL FEMALE
DMBA FEMALE

Figure 8

Figure 9. Densitometric analysis of western blots for cytochromes P4502B, P4502C6 and P4502E1. Samples from adult male (M) or female (F) rats that had been neonatally exposed to diethylstilbestrol (DES) or phenobarbital (PB). See Fig. 7 legend for details.

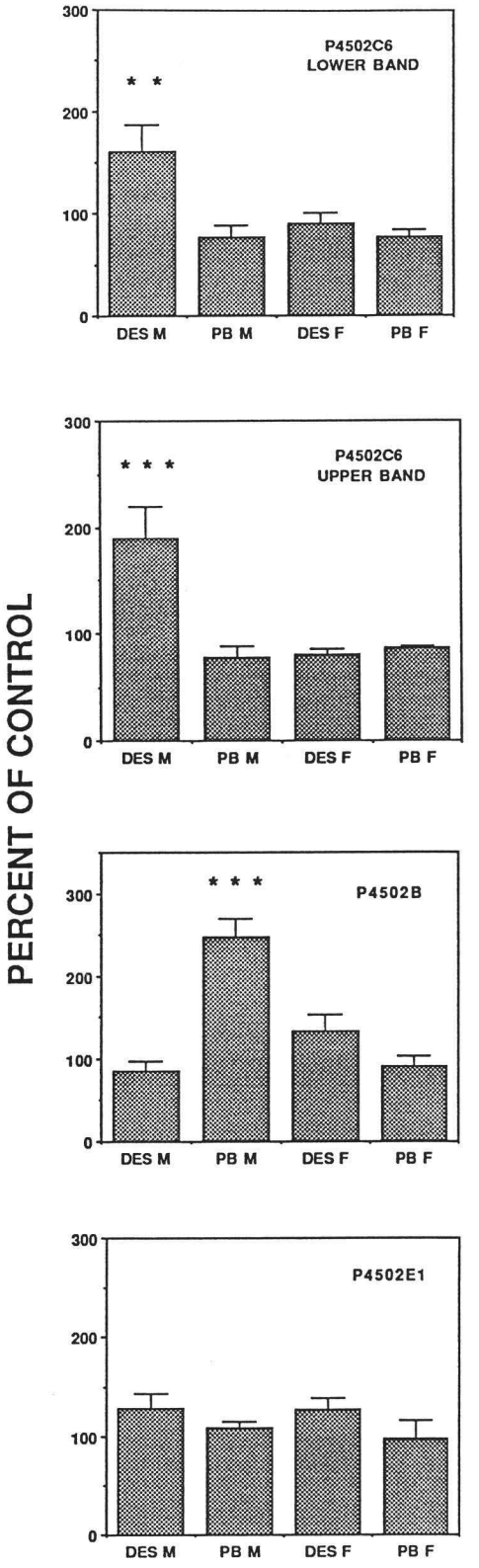
PERCENT OF CONTROL
P4502C6
LOWER BAND
* *
P4502C6
UPPER BAND
* * *
P4502B
* * *
P4502E1
300
200
100
0
DES M
PB M
DES F
PB F

Figure 9

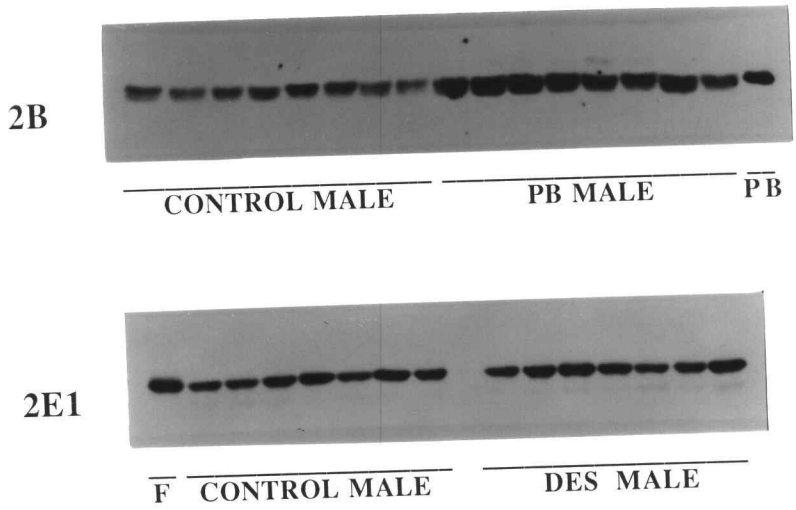

Figure 10. Western blot analysis of hepatic microsomes that had been probed with antibodies for cytochromes P4502B or P4502E1. Samples were loaded at 20 μg/lane except the PB sample which was loaded at 1 μg/lane. KEY: CONTROL MALE - adult males that were neonatally treated with vehicle; PB MALE - adult males that were neonatally treated with phenobarbital; DES MALE - adult males that were neonatally treated with diethylstilbestrol; PB - male induced with phenobarbital for three consecutive days prior to sacrifice.

CHANGES IN METABOLISM OF AFLATOXIN B_1. ALTERATIONS IN α-CLASS GLUTATHIONE S-TRANSFERASES.

Richard C. Zangar

David L. Springer

Jo-Ann McCrary

Raymond F. Novak

Donald R. Buhler

Abstract

Neonatal exposure of rats to xenobiotics has been shown to produce long-term alterations in hepatic enzyme activity and in levels of DNA adducts following carcinogen exposure. We exposed newborn male rats to diethylstilbestrol (DES), pregnenolone-16α-carbonitrile, phenobarbital or 7,12-dimethylbenz(a)anthracene on days 1, 3 and 5 days of age. At five months, males were injected with 1 mg/kg of [^{3}H]-aflatoxin B_1 (AFB1), sacrificed after 2 hours and examined for the formation of AF-DNA adducts in the liver. Males neonatally exposed to DES showed a 35% decrease in formation of DNA adducts. Analysis of the adducted DNA bases failed to show any changes in the DES samples. Hepatic glutathione concentrations were unchanged but a 21% increase in cytosolic protein concentration was observed in the DES males. Protein concentrations of α class glutathione S-transferases (αGST), which are known to inactivate the toxic AFB_1-8,9-epoxide, were more than 2-fold greater than control levels when analyzed using western blot techniques, suggesting that the detoxifying activity of the cytosol may have been increased. To confirm this, in vitro tests were undertaken using induced mouse microsomes to activate [^{3}H]-AFB_1 in the present of treated cytosol and GSH. Analysis of metabolites by HPLC showed that DES treated males formed 245% of the AFB-SG conjugate relative to vehicle controls. These results indicated that neonatal DES treatment resulted

in long term changes in basal αGST levels and that these changes were responsible for lower levels of DNA adduction following adult exposure to AFB_1.

Introduction

Glutathione S-transferases (GSTs[4]) have been shown to play an important role in carcinogenesis by conjugating GSH with electrophilic compounds and thereby preventing binding to cellular macromolecules (for reviews see Sato, 1988; Mannervik and Danielson, 1988; Coles and Ketterer, 1990). Two of the 4 classes of GST are normally found in adult rat liver: the α and the μ classes (αGST and μGST, respectively). Active GST molecules are comprised of two subunits from the same class but not necessarily identical. The hepatic αGST contains the Y_a and the Y_c and these can be clearly separated by SDS-PAGE.

Aflatoxin B1 (AFB_1) is known to be bioactivated by several cytochrome P450 isozymes to the AFB_1-8,9-epoxide, which can bind to DNA in the putative initiating step in carcinogenesis (Shimada et al., 1987; Guengerich, 1977,

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[4]Abbreviations used are GSH - reduced glutathione; GST - glutathione S-transferase; αGST or μGST - α- or μ-class GSTs, respectively; DES - diethylstilbestrol; PB - phenobarbital; PCN - pregnenolone-16α-carbonitrile; DMBA - 7,12-dimethylbenz(a)anthracene, PBS - physiologically buffered saline, PBS-T - PBS plus 0.05% Tween-20; CDNB - 1-chloro-2,4-dinitrobenzene; DCNB - 1,2-dichloro-4-nitrobenzene; SDS-PAGE - sodium dodecyl sulfate polyacrylamide electrophoresis.

1988). Only αGSTs are believed to be able to conjugate AFB_1-8,9-epoxide, forming a nontoxic conjugate that is eliminated in the bile (Ketterer, 1988; Quinn et al., 1990). Differences between induced and control animals and between mammalian species in susceptibility to AFB_1 carcinogenesis have been shown to be the result of differences in αGST conjugation of the AFB_1-8,9-epoxide, even though large differences in bioactivating capabilities may also be present, suggesting that αGST activity may be the predominant factor in determining individual or species susceptibility to AFB_1 (Lotlikar et al., 1984, 1989; Monroe and Eaton, 1987; Degen and Neumann, 1981, Quinn et al., 1990)

Exposure of neonatal rats to xenobiotics such as DES, phenobarbital (PB) and 7,12-dimethylbenz(a)anthracene (DMBA) has been shown to produce long-term changes in hepatic enzymes not seen in adult animals exposed to the same compounds (Bagley and Hayes, 1985b,c; Haake and Safe, 1988; Lamartiniere and Pardo, 1988; Salganik et al., 1980; Zangar et al., unpublished observation). Typically, these effects are delayed until after puberty and may be the result of long-term or permanent changes in the neuroendocrine system in a process that has been termed imprinting (Skett and Gustafsson, 1979). Furthermore, rats exposed <u>in utero</u> or as neonates to phenobarbital (PB) have been shown to have altered levels of DNA adduction as adults following <u>in vivo</u> AFB_1 exposure (Faris and Campbell, 1981, 1983; Bagley and

Hayes, 1985b). Faris and Campbell (1983) showed both increased and decreased binding of AFB_1 to DNA, depending on the dose of PB used.

We examined the long-term effects on AFB_1-DNA adduction levels following neonatal exposure to a synthetic estrogen, DES; a central-nervous-system depressant and general GST inducer, PB; a glucocorticoid, pregnenolone-16α-carbonitrile (PCN); and a polyaromatic hydrocarbon DMBA that is similar in structure to 3-methylcholanthrene, a specific Y_a-subunit GST inducer (Igarashi et al., 1987), but is not as potent a carcinogen. Treatments showing altered AFB-DNA adduction levels were further examined for changes in adduction profile, hepatic GSH concentrations, immunologically detectable levels of αGST, and in the conjugating ability of hepatic cytosolic fractions for activated AFB_1.

Materials and Methods

Chemicals

The [^{3}H]-AFB_1 was obtained from Moravek Biochemicals (Brea, CA) and AFB_1, AFP_1, AFQ_1, AFM_1, reduced and oxidized GSH standards, DES, DMBA, PB, proteinase K, Type-IIA RNase and affinity purified GST were obtained from Sigma. PCN was from Upjohn (Kalamazoo, Michigan). Cold-pressed sesame seed oil was from Hain's.

Animals and accommodations

Pregnant rats were obtained from Charles River (Raleigh, North Carolina) and all pups were born within a single 24 hour period. The day of birth was defined as day 0 of age. Pups were subcutaneously dosed on 1, 3 and 5 day of age with 350 μg/pup/d DES or PCN or 15 mg/kg/d of DMBA in sesame seed oil or with 50 mg/kg/d PB in 0.9% NaCl or vehicle at 3 or 6 μL/g/d for sesame seed oil or saline, respectively. Pups were weaned at 21 days of age and placed in group housing with other animals of the same treatment. Animals were provided free access to Purina rat chow and water during the course of the study.

In vivo binding of AFB_1

Aflatoxin was handled using at least two pairs of gloves, lab coat and protective eyewear in a fume hood. Following use, AFB_1 was detoxified using the procedures of Castegnaro et al. (1981).

At 22 weeks of age, 6 male rats from different litters were selected and injected ip with 200 μL/kg of dimethyl-formamide containing the equivalent of 1 mg AFB_1/kg and 1.4 μCi [^{3}H]AFB_1/kg. (Yu et al, 1988 found that doses up to 3 mg/kg produced a linear response in quantity of DNA adducts formed.) After 2 hours, animals were exsanguinated with sodium pentobarbital. Approximately 1 g sections of the

lower portion of the left liver lobe were removed and cut into thin strips with a razor blade. These strips were then added to 7 mL of 10 mM Tris, 0.1 M NaCl, and 1 mM EDTA and minced into fine pieces with scissors. DNA was then isolated using the procedures of Marmur (1961) as modified by Springer et al. (1989). Concentrations of DNA were determined by absorption at 260 nm using an optical density of 50 μg DNA/mL/absorption unit (Sambrook et al., 1989). Scintillation methods were used to quantify tritiated metabolites adducted to the DNA.

Adduct profiles were determined by digestion of the DNA to individual bases, prepurification of the adducted bases using a C18 SepPak, and HPLC separation followed by fraction collection and scintillation analysis (Croy and Wogan, 1981). The retention times of the 2 major AFB_1 adducts, the 2,3-dihydro-3-hydroxy-(N^7-guanyl)AFB_1 and the AFB_1 formamidopyrimidine adduct - which is an <u>in vivo</u> degradation product of the first adduct (see Essigman et al., 1982), were determined by co-elution with standards generously provided by George Bailey and Pat Loveland. Other peaks were determined by comparing relative elution patterns and peak size with published reports using the same preparative and chromatographic procedures (Croy and Wogan, 1981).

<u>GSH analysis</u>

Seven 24-week-old male rats that had been neonatally

treated with DES or vehicle were anesthetized with an ip injection of sodium pentobarbital and sacrificed immediately after anesthesia. Livers were removed and flushed with cold saline. The right lobe was frozen in liquid nitrogen and stored at -80°C until use. Samples were ground in a mortar and pestle under liquid nitrogen and duplicates of about 50 mg were accurately weighed, placed in 1.5 mL microcentrifuge tubes and stored in liquid nitrogen until use. At no time were samples allowed to thaw. Five hundred μL of perchloric acid (4°C) was added to the frozen tubes, sonicated using a probe sonicator for 10 s and then frozen immediately in liquid nitrogen. Dinitrophenol derivatization of the GSH followed by HPLC analysis was then performed as described (Fariss and Reed, 1987). Levels of derivatized, oxidized GSH (GSSG) were below the level of detection, indicating that less than 1% of the GSH was oxidized during storage and handling.

Western blotting of αGST

Liver samples described in the GSH analysis section were also used in the preparation of cytosolic fractions using the procedures of Primiano et al. (1992). Protein concentrations were determined using the procedures of Bradford (1976) with dye reagent from BioRad. Cytosolic protein was loaded onto a 20 cm SDS-PAGE gel (BioRad apparatus) using a 3% stacking gel and a 12.5% separating

gel as described by Laemmli (1970). Following SDS-PAGE size fractionation, proteins bands were transferred to nitrocellulose (BioRad) using a BioRad electroblotter operated at 30 V for 6 hr in an aqueous solution of 25 mM tris-base, 192 mM glycine and 20% methanol. Nitrocellulose was then dried at 70°C for 30 min, and blocked for 60 min using 15 mL of 5% milk powder (Carnation) in physiologically buffered saline (PBS: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.64 mM KCl, pH 7.4) containing 0.05% Tween-20 (PBS-T). Fifteen μL of affinity purified anti-αGST solution (Primiano and Novak, 1992) was added and incubated for 12 hr. Blots were washed with PBS-T twice for 0.5 and 5 min; incubated with 1:1000 dilution of rabbit-anti-goat horse radish peroxidase conjugate (Sigma) in 5% milk powder, PBS-T for 12 hr; and washed again. Incubations and washes were performed at room temperature with gentle shaking. The enhanced chemiluminescence reagents and procedure of Amersham were used to develop Kodak X-AR5 film using a 2 min exposure period. The density of individual bands was determined using a Zeineh SL densitometer set on the "log" data-integration setting.

Cytosolic GSH conjugation of aflatoxin

Cytosolic conjugation of the activated AFB_1 was assayed using the procedures of Monroe and Eaton (1987) as modified slightly below. In brief, 250 μL samples were pre-incubated

in 80 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 25 mM KCl, 250 mM sucrose, 5 mM GSH, 5 mM glucose-6-phosphate, 0.5 U/mL glucose-6-phosphate dehydrogenase, 2 mM NADPH and 0.33 mg of BHA-induced mouse microsomes (generously provided by David Eaton) at 37°C for 2.5 min followed by the addition of 10 μg of [^{3}H]AFB_1 (2 μCi) and then incubated for 10 min. Incubation were stopped by the addition of 500 μL of 4°C MEOH and mixing. Protein was allowed to precipitate overnight and samples were centrifuged at 12,000 x g for 5 min and the supernatant decanted. 50 μL of supernatant was analyzed by HPLC and an in-line radiometric detector (Radiomatic A-100) and Flo-Scint V scintillant. The HPLC was a Spectra-Physics SP8700 and the column was a Zorbax ODS 4.6 x 250 mm. Mobile phase conditions were: Solvent A - 5% tetrahydrofuran in MEOH (v:v); solvent B - milli-Q filtered water. The mobile phase was at 10% A for the first 2 min, then to 40% A at 4 min, 70% A at 15 min and 100% A at 20 min. All gradients were linear.

Statistics

Data from treated animals were compared to the appropriate vehicle control using an unpaired, two-tailed Student's t-test with a significance level of $p < 0.05$ (Zar, 1974).

Results

Adduction levels to DNA by [^{3}H]AFB_1 metabolites were reduced to 65% in adult males that had been treated with DES as neonates (Fig. 11). Other treatments did not significantly alter adduction levels and, hence, were not examined further. The AFB_1-adducted DNA from the DES and vehicle control animals was then digested to single bases and analyzed by HPLC. Results from this procedure indicated that there was no change in the adduction profile when the frequency of the individual adducts was observed on a relative basis (Fig. 12). We felt that these results were more consistent with a shift in phase II than in phase I metabolism and proceeded to analyze for changes in GSH concentrations and GST activities. Hepatic concentrations of GSH were not altered in the DES males (mean ± SE for vehicle- or DES-treated males were 5.29 ± 0.50 or 5.25 ± 0.32, respectively). However, cytosolic protein recovery was increased 22% in the DES males relative to the control animals (mean ± SE for vehicle or DES-treated males were 20.0 ± 0.71 and 24.3 ± 1.72, respectively).

Western blot analysis using antibodies specific for the α-class GST detected 3 bands in the treated samples and in affinity purified fractions of GST (Fig. 13). Four bands were observable in affinity purified fractions of rat liver GST stained with Coomassie Blue. Based on reported SDS-PAGE migration of hepatic GSTs (Primiano et al., 1992; Igarashi

et al., 1985; Sato, 1988; Mannervik and Danielson, 1988), the upper band corresponds to Y_c (M_r 28,000), Y_b/Y_b' (M_r 26,500) and the 2 lowest bands, both of which migrated in close proximity, were where Y_a (Mr 25,000) would be expected (Fig. 13). Subunit Y_a can be separated into 2 bands by HPLC (Coles and Ketterer, 1990) and it is possible that both forms are being detected here. Subunit Y_k, which is also present in rat liver, belongs to the α class and has a Mr of 24,500, could also account for the lower of the two bands. However, levels of Y_k are normally very low (Coles and Ketterer, 1990) and would not be expected to be detected here. To be consist with the published literature, we will refer to these 2 closely migrating bands simply as the Y_a bands. The Y_c and Y_a bands were detected by antibodies for the αGST but not the Y_b/Y_b' band, as expected. Western blot analysis showed that the Y_c band, which was much more intense than the Y_a bands, was increased to 222% of the control values (Table 2). The two Y_a bands, barely visible in Fig. 13 (exposure was limited to prevent overexposure of the upper bands) could not be cleanly separated by the densitometer. However, visual observation indicated that relative levels of all three bands were coordinately regulated in individuals. Densitometric analysis of these combined Y_a bands indicated that DES males had 234% of control levels, similar to the upper band.

In order to assess the <u>in vitro</u> glutathione conjugating activity of the imprinted cytosols, AFB_1 was converted to

the AFB_1-8,9-epoxide using BHA-induced mouse microsomes, which have been shown to be an efficient epoxide generating system (Monroe and Eaton, 1987). Analysis of metabolites by HPLC showed that the AFB-SG conjugate was increased in the neonatally treated DES males to 245% relative to control animals (Table 2). Other metabolites were unchanged between treatments (e.g. mean ± SE for AFP_1 levels were 159 ± 10.8 and 167 ± 4.3 for control and DES samples, respectively).

Discussion

We observed a 35% decrease in adduction levels in neonatally treated DES males. Further, these changes could be associated with increased levels of αGST protein and conjugating activity. The increase in cytosolic protein concentration observed in the DES males may, at least in part, reflect the increased levels of GST, which normally account for about 10% of total cytosolic protein (Reed, 1987).

The increase in αGST immunoreactive protein and the associated AFB-SG conjugating activity in the DES treated males appeared to be a sex-related shift. Females had higher levels in both of these parameters (Table 2) suggesting that neonatal DES resulted in a shift towards the female pattern of metabolism. Female rats have been shown to have higher levels of αGST subunits but these differences

are not apparent until about 7 or 8 weeks of age (Igarashi et al., 1985, 1987; Rogiers, 1991). The development of α-class subunit female-predominance coincides with the onset of puberty and is consistent with neuroendocrine-mediated regulation of the GSTs. This suggests that the neonatal DES may be acting on the neuroendocrine system rather than directly on the liver. The five month delay between exposure and analysis is consistent with developmental changes in regulatory processes rather than direct or short-term inductive changes.

Lamartiniere and Pardo (1988) are the only researchers to examine GSTs in imprinted animals prior to this study. They examined adult GST enzymatic activities using 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) following neonatal treatment with DES. These researchers did not observe alterations in basal levels in males although significant changes were observed in GST activities following induction by PB. However, DCNB is metabolized primarily by the μ-class of GSTs (Y_b and Y_b') and CDNB is conjugated at similar levels by all hepatic forms (Mannervik and Jensson, 1982). Igarashi et al (1987) also have shown that although adult females had greater levels of the αGST subunits, GSH conjugation with DCNB or CDNB was greater in male cytosolic fractions. These latter two studies suggest that substrate analyses using CDNB and DCNB may not be adequate to detect changes in αGSTs.

Acknowledgements

The authors would like to thank Donald Reed and Paul Jean for assistance in the GSH analysis. This research was supported by the U.S. Department of Energy through the Northwest College and University Association of Science (Washington State University, grant DE-FG06-89ER-75522) and by NIH (grant ES-00210).

Table 2. Results of αGST immunoblot analysis and <u>in vitro</u> conjugation of AFB_1-8,9-epoxide.

	Control Male	DES Male	Control Female
Density[a]	100 ± 20[b]	222 ± 27**	207 ± 32*
pmol/mg/min[c]	50 ± 6	123 ± 26**	244 ± 42***

[a]Laser densitometry results of the Y_c band shown in the western blot in figure 13 and a similar blot prepared with 6 control females and 6 control males. Values as a percent of control male.
[b]Mean ± SE.
[c]Pmol of AFB-SG conjugate formed per mg of cytosolic protein. Values determined from 7 males or 3 females per treatment.
*, ** or *** - Significantly different from control values at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively.

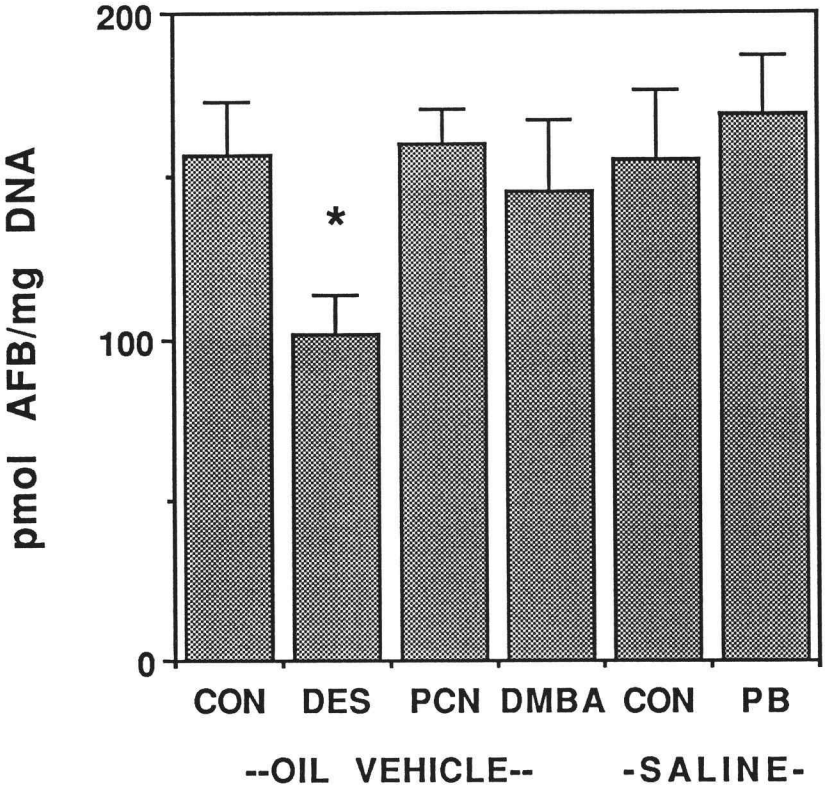

Figure 11. In vivo binding of [^{3}H]AFB$_1$ metabolites to DNA. Analysis of 22-week old male rats that had been neonatally exposed to diethylstilbestrol (DES), 7,12-dimethylbenz(a)anthracene (DMBA), pregnenolone-16α-carbonitrile (PCN), phenobarbital (PB) or vehicle (OIL or SALINE). Columns and crossbars represent mean and SE, respectively, of 6 individuals. * - significantly different from vehicle control at $p < 0.05$.

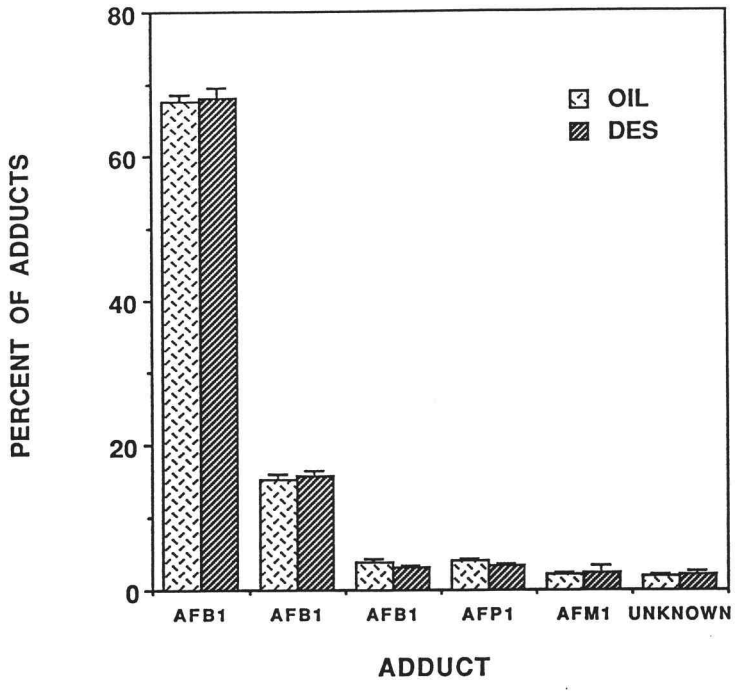

Figure 12. Profile of individual [^{3}H]aflatoxin adducts to DNA bases. Samples from male rats neonatally treated with diethylstilbestrol (DES) or vehicle (OIL) that were then exposed to [^{3}H]AFB_1 <u>in vivo</u> as adults. DNA was digested to individual bases and analyzed by HPLC/scintillation. Columns and cross bars represent means and SE, respectively, of individual peaks as a percent of total adducts within the sample. The AFB_1, AFP_1 or AFM_1 represent the appropriate 8,9-epoxide which formed the adduct with guanine at the N^7 position. The presence of several AFB-N^7-guanine peaks reflects the nonenzymatic rearrangement of this adduct <u>in vivo</u> (see Essigmann et al., 1982).

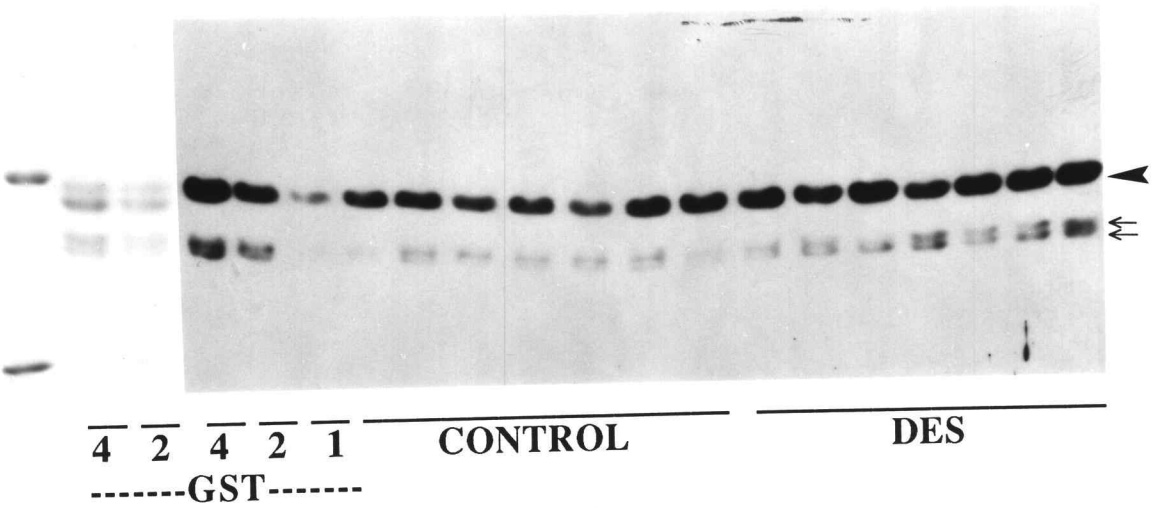

Figure 13. Western blot of α class glutathione S-transferases. Blot was prepared using cytosolic fractions and αGST-specific antibodies and an identically prepared gel stained with Coomassie Blue (shown in the first three lanes on the left). Male rats that had been neonatally treated with diethylstilbestrol (DES) or vehicle were examined at 24 weeks of age. The "GST" samples represents 4, 2 and 1 μg of affinity purified GST fraction isolated from rat liver. The first lane contains molecular weight standards of carbonic anhydrase (30,000; upper band) and trypsin inhibitor (20,100; lower band). The upper band (arrowhead) corresponds to the Y_c subunit while the lower two bands (small arrows) both migrate near where the Y_a subunit is expected. See text for details.

SUMMARY AND CONCLUSIONS

Results indicated that neonatal exposure to DES, PB or DMBA altered in hepatic enzyme levels in adult rats at 5 months of age. The 5 month delay between dosing and examination was consistent with long-term changes in regulatory processes rather than direct actions of the compounds themselves.

Cytochrome P450 enzymes found to be altered in this study are potentially of great importance in the normal physiological status of the animal. Cytochrome P4502C11 was found to be altered in some treatments in this study and these changes could be related to altered testosterone metabolism. This P450 also hydroxylates cholesterol at the 7α position, the putative rate-limiting step in bile acid synthesis; hydroxylates 1α-hydroxy-vitamin D_3 at the 25 position; and has high arachidonic acid epoxygenase activity, converting arachidonic acid to physiologically active forms (Nguyen et al., 1990; Eldredge et al., 1989; Guengerich, 1986; Cadevilla et al., 1990). Disruption of hepatic P450 levels clearly has the potential to have far reaching effects on normal physiological homeostasis. Changes in these enzymes may also be expected to have profound effects on the metabolism, excretion and toxicity of a wide variety of foreign compounds including drugs, pesticides and carcinogens (for reviews see Guengerich, 1986, 1990). Glutathione transferases were also found to be

altered in this study and this was related to altered metabolism and DNA adduction of aflatoxin B_1. However, these enzymes also are very important in the metabolism and excretion of electrophilic compounds and maintaining proper redox status in the cell (for reviews see Reed, 1989; Mannervik and Danielson, 1988).

Neonatal DES appeared to have the greatest effect on adult metabolism. Changes were observed in 6 of the 10 hepatic enzymes analyzed (Table 3) and in hormone and carcinogen metabolism. In general, these changes could be interpreted as a feminization process, consistent with steroidal imprinting of the hypothalamus as modeled by Skett and Gustafsson (1979). Increases in female-predominant forms cytochrome P4502A1, cytochrome P4502C12 and αGSTs were observed combined with decreases in male-specific forms P4502C11 and P4503A2. In the case of the male-specific forms altered by DES, the response was highly variable with some animals showing dramatic decreases in enzyme levels while others did not appear to be strongly affected. This suggests that in males there was a threshold level, either in response to dosing or in the tissue response in the adult, and that once this threshold was reached there was a dramatic "switching" from the masculine to the feminine regulation. Interestingly, male-specific form P4502C13 was not altered in the DES-treated males, even in rats with virtually no P4502C11 or P4503A2. Feminine patterns of growth hormone have been shown to inhibit P4502C13 while

masculine secretion patterns are required to induce the P4502C11 and P4503A2 (McClellan-Green et al., 1989). These results suggest that the strongly affected DES males had neither typical male nor female patterns of GH secretion. Neonatal DES also altered levels of P4502C6 and a second similar form that may be P4502C7, both of which did not show sex-specificity. It is not likely that either of these two enzymes is strongly regulated by GH and therefore changes in these enzymes would not be predicted by current models of imprinting.

Neonatal DMBA only altered P4502C12 (Table 3). The increase in this P450 was similar to what was seen with neonatal DES. Since both of these compounds have known estrogenic actions, it seems likely that both are working in a similar manner. The fact that DES was a much more effective imprinting agent may be related to the higher dose used for this compound or unknown potency differences in the compounds. The fact that DMBA only altered P4502C12 may indicate that this form is especially susceptible to imprinting by estrogen-like compounds.

Neonatal PB increased levels of P4502C11 and P4502B. These effects are distinct from those observed with DES or DMBA and may be indicative of a different mechanism of imprinting by PB. The possibility that these changes are in some way a direct result of PB induction can not be ruled out since both enzymes can be induced by PB. However, other PB-inducible forms, such as the P4503A2 and P4502C6, were

not imprinted by the neonatal PB. Also, PCN induces P4502B, P4503A1 and P4503A2 but was not found to act as an imprinting agent in this study. It seems likely that the regulatory processes involved in the long-term changes observed here are not the same as observed following induction in the adult. Instead, it seems more likely that PB imprinting was the result of interaction of PB and steroid hormones. Arai and Gorski (1968) found that PB inhibited masculinization by exogenous androgen in female rats. Bagley and Hayes (1985c) also found that testosterone modified PB imprinting. In castrated males, neonatal PB resulted in greater changes in hepatic enzyme activity while additional dosing with TP reduced the degree of imprinting.

Table 3. Summary of changes in hepatic enzymes in adult rats neonatally exposed to DES, PB of DMBA.

P450	DES		PB		DMBA	
	Male	Female	Male	Female	Male	Female
2A1	158[a]	NS	NS	NS	NS	NS
2B	NS	NS	250	NS	-	-
2C6	160	NS	NS	NS	-	-
2C7[b]	190	NS	NS	NS	-	-
2C11	58	-	127	-	NS	-
2C12	-	138	-	NS	-	148
2C13	NS	-	NS	-	NS	-
2E1	NS	NS	NS	NS	-	-
3A2	60	-	NS	-	NS	-
αGST	222	-	NS	-	-	-

[a]Significantly different mean values from enzymatic or western blot are shown as a percent of vehicle control values. NS - no significant difference. Dash indicates that no analysis performed for that treatment.
[b]"P4502C7" was the upper band detected by anti-P4502C6 in western analysis and was not definitively identified. Significantly different mean values from enzymatic or western blot are shown as a percent of vehicle control values. NS - no significant difference.

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